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Increasing ARCAT (Trade Name) Test Sensitivity for Examination of Potable Waters

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INCREASING ARCAT® TEST SENSITIVITY FOR EXAMINATION OF POTABLE WATERS

by

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16. ABSTRACT

The primary objective of this research project was to increase the sensitivity of the ARCAT method to detect one coliphage per 100 ml of water sample. Several concentration techniques were evaluated and three methods selected for in-depth study. Two of the methods selected for in-depth study were filter concentration techniques. The third method involved amplification of coliphages by addition of nutrients and a host culture followed by incubation to allow a lytic burst cycle to occur. Of these techniques, the Zeta Plus 60S filtration technique proved to be the optimum method. Coliphage adsorption with this filter range from 80-100%. Coliphage recoveries from the filters were ≥90%. With this concentration method, test results can be obtained within 6 1/2 hours with a sensitivity of 1 coliphage per 100 ml.

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FOREWORD

The U.S. Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water, and spoiled land are tragic testimonies to the deterioration of our natural environment. The complexity of that environment and the interplay of its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution; it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems to prevent, treat, and manage wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, to preserve and treat public drinking water supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research and provides a most vital communications link between the researcher and the user community.

This report evaluates concentration methods for increasing the sensitivity of the ARCAT® coliphage detection procedure for use as a rapid test of potable water quality and water treatment efficiency. Relationships between changes in total and fecal coliform and coliphage parameters resulting from physical treatment of potable source water are also reported.

Francis T. Mayo, Director
Municipal Environmental Research
Laboratory

ABSTRACT

The primary objective of this research project was to increase the sensitivity of the ARCAT method to detect 1 bacteriophage per 100 ml of water sample. Seven concentration techniques were evaluated, and three methods were selected for in-depth study. Two of the methods selected were filtration methods using positively charged and negatively charged filters. The third technique amplified bacteriophages by addition of nutrients and host culture followed by incubation to allow for a lytic burst to occur.

Both filtration methods demonstrated good bacteriophage adsorption, and recoveries of greater than 70%. The positively charged filters were selected as the filtration method of choice based on the time required for filtration and consistency of bacteriophage recovery. The Zeta Plus 60S filter had the best adsorption and recovery characteristics of all the Zeta Plus filters evaluated. Optimum adsorption of bacteriophages on this positively charged filter (80% to 100%) was obtained when the pH of the water sample was adjusted to 6.0 before filtration. Three percent trypticase soy broth adjusted to pH 8.5 gave the highest bacteriophage recoveries ($\geq$90%). The concentration method using the Zeta Plus 60S filter was compared with plating all 100 ml of the water sample using the standard ARCAT. Both natural waters and sterile waters spiked with known concentrations of bacteriophages were evaluated in this comparison. The natural water data showed more variability than did the spiked samples of a single bacteriophage type. Least squares linear regression analysis of natural waters concentration data indicated a highly significant regression with a calculated F ratio from the analysis of variance of less than 0.00001. Thus with the Zeta Plus 60S filtration technique, bacteriophage levels of 1 per 100 ml of sample can be reliably detected in as little as 6 1/2 hours at a cost of $4.12.

Optimization of the lytic burst for amplification of bacteriophages was accomplished by addition of nutrients and *E. coli* C host culture to 1×10^8 colony forming units/ml in the sample, followed by incubation at 35°C for 70 minutes before plating of four 5 ml aliquots by the standard ARCAT technique. This amplification method detects the presence of very low levels of contamination in water samples, but it does not give precise numbers of plaque forming units in the original sample. A significant amount of research on bacteriophages present in natural waters and their plaque morphology, burst sizes, and burst times remains to be done before the method will yield quantitative results.

Examination of the effects of physical treatment of water indicated that settling and filtration are effective means for reducing levels of bacteriophages and total and fecal coliforms in natural raw waters. Total and fecal coliforms were reduced by approximately 99% and 97%, respectively, whereas bacteriophages were less efficiently removed (approximately 93%) by these processes.

Disinfection studies indicated that chlorine dioxide was effective as a viricide and bacteriocide. At the ozone levels achieved during this study, bacteriophages and total or fecal coliform levels were not consistently reduced. Ozone disinfection effectiveness was both concentration and exposure time dependent.

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LIST OF ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

AF	- antifoam agent
$Al(OH)_3$	- aluminum hydroxide
$Al_2(SO_4)_3$	- aluminum sulfate
ARC	- Atlantic Research Corporation
ARCAT	- A Rapid Coliphage Analysis Technique
ASTM	- American Society for Testing and Materials
ATCC	- American Type Culture Collection
B.E.	- beef extract
$CaCl_2$	- calcium chloride
$Ca(OH)_2$	- calcium hydroxide
Ca_3PO_4	- calcium phosphate
cfu	- colony forming unit(s)
cm	- centimeter
E. coli	- Escherichia coli
FC	- fecal coliform
g	- gram
HCl	- hydrochloric acid
hr	- hour(s)
KCl	- potassium chloride
L	- liter(s)
M	- molar
mg	- milligram(s)
$MgCl_2$	- magnesium chloride
min	- minute(s)
ml	- milliliter(s)
mm	- millimeter(s)
MNA	- modified nutrient agar
m.o.i.	- multiplicity of infection
NA	- nutrient agar
NaCl	- sodium chloride
NaOH	- sodium hydroxide
Na_3PO_4	- sodium phosphate
NB	- nutrient broth
NH_4NO_3	- ammonium nitrate
nm	- nanometer
NMR	- nuclear magnetic resonance
O_3	- ozone
O.D.	- optical density
pfu	- plaque forming unit(s)
pH	- hydrogen ion concentration

ppm	- parts per million
PTI	- Public Technology Inc.
sp	- species
$Sr(NO_3)_2$	- strontium nitrate
TC	- total coliforms
TNTC	- too numerous to count
TSA	- trypticase soy agar
TSB	- trypticase soy broth
UV	- ultraviolet
v/v	- volume per volume
w/v	- weight per volume

SYMBOLS

10X	- ten times the usual concentration
$\geq$	- greater than or equal to
$\leq$	- less than or equal to
μm	- micrometer
$\sim$	- approximately
Φ	- bacteriophage
^oC	- degrees centigrade

SECTION 1

INTRODUCTION

Waterborne diseases can be spread rapidly by bacterial and/or viral contaminants contained in any waters used for human consumption. To prevent the spread of these diseases, community water supplies are treated to remove or reduce these contaminants by such methods as settling, filtration, and disinfection. Standard tests (i.e. membrane filtration or most probable number) for detection of total coliform bacteria (indicators of sanitary quality) are routine procedures for determining the treatment efficiency. Results from these procedures are not available for 24 + 2 hours. Water treatment plants use chlorine as a means of disinfection (i.e. to inactivate harmful organisms). A chlorine residual is maintained in the distribution system to provide biocidal material throughout the entire system. A rapid test to evaluate the sanitary quality of the water could impact on the decision of whether or not additional treatment, such as booster chlorination in the system, is necessary.

Under contracts with the U.S. Department of the Army (DAAOS-69-D-0134 DAADOS-71-C-0177), Atlantic Research Corporation (ARC) developed a rapid test for determining the presence of *Shigella* sp., *Salmonella* sp., and *Escherichia coli* in water. The test determined the number of bacteriophages of a given bacterial species in a water sample within 4 to 6 hours. The detection of *E. coli*, the coliform bacterium used as an indicator of fecal contamination, was of particular interest. A quantitative relationship between coliform bacteria and their coliphages was established as a result of this work (Kenard and Valentine, 1974).

ARC, in cooperation with Public Technology, Inc. (PTI), initiated field tests in 10 U.S. cities for the coliphage method, ARCAT® (A Rapid Coliphage Analysis Technique). A second field test program was conducted in 1978. This field test was jointly sponsored by PTI, ARC, and the 12 participating U.S. cities. The field tests confirmed that the ARCAT procedure is easy and inexpensive to perform and gives rapid reliable results. The quantitative relationship between coliphage and coliforms established in earlier studies was shown to be valid for natural waters sampled throughout the United States (Wentsel *et al.*, 1981).

Under a National Science Foundation Grant (Scott *et al.*), further evaluation of the use of bacteriophages as indicators of fecal pollution was conducted. During this study, the test procedure was simplified, the conditions for coliphage replication and plaque formation optimized, and the influence of chlorine and other disinfectants on the coliphage-coliform relationship was evaluated. The sensitivity of the simplified ARCAT test was improved over that observed previously, but it was not suitable for determination of water quality in the potable water range.

The present study focused on increasing the sensitivity of the ARCAT test to the level of 1 bacteriophage per 100 ml. A secondary objective was evaluation of the relative effects of water treatment processes on bacteria and bacteriophages.

SECTION 2

CONCLUSIONS

The primary objective of this study has been achieved: to increase the sensitivity of the ARCAT® (A Rapid Coliphage Analysis Technique) method for detection of 1 bacteriophage per 100 ml of water sample. The following concentration techniques aimed at achieving this objective were initially evaluated in a feasibility study:

- Concentration by filtration through positively charged filters
- Concentration by filtration through negatively charged filters
- Osmotic filtration (dialysis)
- Controlled lytic bursts
- Adsorption on inorganic salts
- Polymer two-phase separation
- Magnetic filtration

Feasibility studies of an additional method, electrophoresis, were abandoned when the electrophoresis apparatus failed to perform satisfactorily and other techniques appeared more promising.

Three methods were selected for in-depth study based on recovery of bacteriophages, time required for availability of results, and ease of performing the technique. Two of the methods selected were membrane filtration methods that yielded good bacteriophage adsorption and recovery. These methods used positively charged and negatively charged filters, and added 0.5 and 1.5 hours, respectively, to the time required for the ARCAT method. The third method evaluated was amplification of bacteriophages by incubation to allow for a lytic burst to occur. Amplification increased the time required for availability of test results by 70 minutes.

The concentration efficiencies of the positively and negatively charged filter methods were evaluated using sterilized water samples seeded with known concentrations of a stock bacteriophage. Both filter types demonstrated good adsorption and bacteriophage recoveries of greater than 70% were obtained. The positively charged and negatively charged membrane filter concentration techniques were optimized with respect to bacteriophage adsorption and elution conditions. However, because of occasional clogging of the negatively charged filters and the additional hour required to con-

centrate the sample with the negatively charged filter, the positively charged filter was selected as the concentration method of choice. The Cuno Zeta Plus 60S positively charged filter consistently had the best bacteriophage adsorption (80-100%) and recoveries (≥90%). This filter performed best when the pH of the water sample was adjusted to pH 6.0 before filtration. Recoveries of the bacteriophages from the filter were consistently high when 3% Trypticase Soy broth adjusted to pH 8.5 was used as the eluent.

The positively charged filter concentration technique using the Zeta Plus 60S filter was evaluated using water samples from the Fairfax Water Treatment Plant intake and settled water before chlorination. Bacteriophage recoveries by the positively charged filter concentration technique were compared with bacteriophage recoveries obtained by plating 100 ml of the water sample by the standard ARCAT procedure. The recoveries of bacteriophages from natural water samples were more viable than those obtained from spiked samples. Least square linear regression analysis was used to determine how well the Zeta Plus concentration method predicted the number of bacteriophages originally present in the natural water sample. The regression was highly significant with a calculated F ratio from the analysis of variance of less than 0.00001. The coefficient of determination was 0.8516, indicating that more than 85% of the variation in the bacteriophage deterination was related to the concentration of bacteriophages filtered.

Detection of low level (1-5 pfu/100 ml) bacteriophage contamination in water samples was accomplished by amplification of bacteriophage(s) present by lytic bursts. Based on data obtained in these studies, water samples of 100 ml or 200 ml were suitable for amplification. Nutrient addition was required. E. coli C host as a fresh suspension or a thawed suspension of a frozen preparation could be used to yield a final concentration of 1 x 10^8 cfu E. coli C host cells/ml in the amplification procedure. This concentration of E. coli C provided optimal conditions for adsorption of the bacteriophages onto the host cells, bacteriophage replication and lytic bursts. Incubation for 70 minutes at 35°C provided optimum amplification of bacteriophage types for which E. coli C is a suitable host. The amplification technique was found to be most valuable when used in conjunction with the standard ARCAT procedure for waters expected to contain less than 6 bacteriophages/100 ml (the detection limit for the ARCAT procedure). If bacteriophages were not found by the standard ARCAT procedure (less than 5 bacteriophages present in the sample), the amplification technique allowed the user to deterine if any bacteriophages were present. This amplification technique was found to yield only qualitative results at the 1-5 bacteriophage/100 ml level. Quantitation could be achieved with the amplification procedure if more information on bacteriophages found in raw and finished water was known.

Physical treatment of water (settling, filtration) reduced bacteriophages, total and fecal coliforms by 90-100%. In a brief study of the effects of UV, chlorine dioxide and ozone disinfectants, it was found that bacteriophages were as sensitive to ultraviolet light as total and fecal coliforms. At a concentration of 5 mg/L chlorine dioxide was effective as a

viricide and in reducing fecal and total coliforms. At the ozone levels achieved during this study bacteriophages and total or fecal coliform levels were not consistently reduced. Ozone disinfection effectiveness was both concentration and exposure time dependent.

In summary, the use of the Zeta Plus 60S positively charged filter to concentrate low levels of bacteriophages in a water sample provided the increase in ARCAT test sensitivity needed for routine examination of potable waters for bacteriophage. Test results were obtained in 6 1/2 hours at an approximate cost of $4.12 per test. The ARCAT results can be used to estimate the number of fecal and total coliforms in the water sample or may be used independently as an indicator of water quality.

SECTION 3

RECOMMENDATIONS

1. The ARCAT method using the Zeta Plus 60S concentration technique has now been developed to the point where it can be used to determine the quality of potable waters. The next step in the development of this method is evaluation under actual water quality laboratory conditions. Therefore, field testing of the method in water quality laboratories throughout the United States should be conducted. The laboratories selected for this testing should be chosen so that a wide variation in water samples, treatment processes and testing expertise can be evaluated. Each laboratory should evaluate at least 50 samples over a year's time so that the widest variety of environmental conditions can be evaluated. The following data should be obtained on each sample and sent to a central laboratory for evaluation: 1) water pH and turbidity, 2) number of milliliters filtered, 3) number of bacteriophages present, 4) number of fecal and total coliforms present, and 5) virus data, if available.

2. Amplification of bacteriophages in natural water samples will detect low level contamination but does not allow for quantification of the actual pfu present in the original sample. Further study of bacteriophage morphology, plaque size, burst size and the significance of the presence of any one bacteriophage type in a water sample is needed. For this type of study, isolation and identification of bacteriophages present in sewage and potable waters must first be accomplished. These individual bacteriophages should then be studied to determine the optimum host, burst size and plaque morphology. After recognition and quantitation techniques are developed, sampling of water supplies for these bacteriophages and a correlation with epidemiological information should yield a predictive test for waterborne disease outbreak.

3. An increasing amount of evidence indicates that fecal and total coliforms are not good indicators of water quality. The main problem with the use of the coliform bacteria as indicators of water quality stems from their inability to survive the water physical treatment and disinfection processes as well as enteric viruses. Bacteriophages survive physical water treatment processes better than the coliform bacteria. A study to correlate virus and bacteriophage survival through water treatment processes is recommended. This study should initially select one or two viruses and bacteriophages to be monitored and should subject them to physical treatment process and disinfection processes under controlled conditions. Once survival data have been obtained under controlled conditions, survival of the viruses and bacteriophages should be evaluated under actual treatment plant conditions.

SECTION 4

MATERIALS AND METHODS

MATERIALS

Equipment

Equipment used in performance of the standard ARCAT and in the ARCAT concentration method is listed below:

- Circulating water bath, Julabo Paratherm IV (44.5 $\pm$ 0.2°C)
- Incubator, Precision (35 $\pm$ 0.5°C)
- Water bath, Fisher Versabath (37 $\pm$ 0.5°C)
- Petri dishes, General Scientific, 100 x 15 mm
- Pipets, sterile, 5 ml and 25 ml
- Test tubes with caps, 16 x 125 mm and 20 x 150 mm
- Bausch & Lomb Spectronic 20, 530 nm
- Hach Ratio Turbidimeter
- Fisher Acumet pH Meter, Model 140A

Reagents

Reagents used in performance of the standard ARCAT and in the ARCAT concentration method are listed below:

- Reagent grade chemicals were used in all tests
- Glass distilled water was used in all media
- Host culture was American Type Culture Collection culture #13706 (*Escherichia coli* C)
- Modified Nutrient Agar - formulated as follows:

Nutrient Agar (Difco Bacto)	23.0 g
NaCl	5.0 g
$Sr(NO_3)_2$	2.33 g
NH_4NO_3	1.76 g
water	1 liter

- NaOH - 1 M, 0.1 M for adjusting pH
- HCl - 1 M, 0.1 M for adjusting pH
- Glycine - Nutritional Biochemical Corporation
- Glycerine
- Trypticase Soy Broth (TSB)
- Beef extract - 3%, BBL
- Antifoam B emulsion - Dow Corning

Reagents and materials used in the concentration techniques are listed below:

- Zeta Plus Filters (50S, 60S, 70C, B0204-1MDS) - AMF Cuno, Meriden, Ct.
- Cox Glass Filter (2 μm, 0.45 μm) - Cox Instruments, Detroit, Mich.
- Bio-Rad Nitrocellulose Filters - Bio-Rad Laboratories, Richmond, Ca.
- Magnesium chloride
- Dialysis tubing - A. H. Thomas
- Carbowax PEG 6000 - Fisher Scientific
- Aluminum hydroxide
- Calcium phosphate
- Calcium hydroxide
- Bio-Rad unipore disc prefilter - Bio-Rad Laboratories, Richmond, Ca.
- Tween 80 - Fisher Scientific Co.
- Sodium phosphate
- Sodium chloride
- Dextran sulfate (sodium salt) - BDH Biochemicals LTD., Gallard-Schlesinger Chemical Corp., Carle Place, N.Y.

- Potassium chloride
- Iron ore magnetite - (20 mesh) Iron Mtn. Mines Inc.
- Calcium chloride
- Aluminum sulfate
- Gauss magnet - Varian MNR component
- M-Endo Agar LES - BBL, Fisher Scientific Co.
- Gelman GN-6 0.45 μm filters - Fisher Scientific Co.
- Magnesium sulfate
- M-FC agar - Difco, Fisher Scientific Co.
- Brilliant Green Bile Broth (BGB)
- Rosolic acid
- Lauryl Tryptose Broth (LTS)
- Shortwave ultraviolet lamp - GE, G30T8, 30 watt
- Sodium chlorite
- Sulfuric acid
- Sodium thiosulfate
- Potassium iodide
- Starch - Fisher Scientific
- Hach Ozone Test Kit model OZ-2 - Hach Chemical Co., Loveland, Colo.
- High Range Chlorine Test Kit, model CN-2IP - Hach Chemical Co.
- Hach Free and Total Chlorine Test Kit (0-3 mg/l), model CN-66
- Fisher Variac, model 116 - Fisher Scientific

Stock Bacteriophage Cultures

Bacteriophage cultures used in evaluation of the ARCAT method and the ARCAT concentration method are listed below:

- T3
- ΦX174

- T2 (ATCC #11303B_2)

- ARC #1: isolated from a water sample collected from Turkeycock Run 3/14/77 and grown on nutrient agar plates with E. coli C host strain (ATCC #13706). Bacteriophages were picked from the plate, grown on E. coli C #13706 sterile filtered, and freeze dried.

METHODS

Sampling

One liter glass bottles with ground glass stoppers sterilized in a 170°C oven for no less than 2 hours were used for collection of all samples. All samples collected were grab samples. Samples from creeks and ponds were collected as close to the center of flow as possible. Samples collected from water treatment plants were collected from constantly flowing taps. Samples were processed within four hours of collection.

Specific sample sites are listed below:

- Fairfax County Water Treatment Plant (Occoquan Reservoir) - sample collected from the constantly flowing influent tap.

- Fairfax County Water Treatment Plant - sample of settled water collected from a depth of 1.5 feet below the surface of the settling basin.

- Fairfax County Water Treatment Plant - sample of filtered water collected from a tap in the lower level of the facility before the chlorination site.

- Cherokee Creek - sample collected midstream under the bridge where Cherokee Avenue crosses the creek.

- Accotink Creek - sample collected midstream several hundred yards below the dam.

- Potomac River Water Filtration Plant - sample of raw Potomac River water collected from a constantly running tap of east or west influent water.

- Potomac River Water Filtration Plant - sample of settled Potomac River water collected from a constantly running tap labeled filter influent.

- Potomac River Water Filtration Plant - sample of filtered Potomac River water collected from a constantly running tap labeled filtered water before chlorination takes place.

ARCAT Procedures

Frozen Host Preparation —

The frozen host is prepared for use in ARCAT as follows:

- Inoculate two 100 ml volumes of sterile trypticase soy broth (TSB) containing 10% (w/v) glycerine with a loop of E. coli C culture and incubate 18 hours at 35 ± 0.5°C.

- Inoculate each of two 900 ml volumes of TSB containing 10% glycerine with the 100 ml volumes of E. coli C culture prepared above and incubate at 35 ± 0.5°C until an optical density of 0.4 to 0.55 at 530 nm (equivalent to 10^9 E. coli cells/ml) is obtained on the Spectronic 20.

- Combine the 2 samples and chill to 9°C.

- Pipet 4.5 ml aliquots of the E. coli C culture at O.D. 0.4-0.55 into sterile vials.

- Cap the vials. Freeze the vials at -20°C. (A "frost-free" freezer is undesirable because of thawing cycles which result in loss of host culture viability within 14 days).

Standard ARCAT Procedure —

The ARCAT Procedure A is used for analysis of water samples containing less than 1,000 coliphages/100 ml of water sample. The procedure is presented below:

- Thaw a vial(s) of frozen host in a 44.5 ± 0.2°C water bath for 15 minutes. One vial of host culture is needed for each water sample.

- Approximately 25 ml of water sample are placed in a sterile test tube. The test tube is placed in the 44.5 ± 0.2°C water bath for 5 minutes to permit temperature equilibration and prevent premature agar solidification when agar and sample are combined.

- Four tubes containing modified Nutrient Agar (MNA) (5.5 ml) are placed in boiling water or flowing steam to melt the agar. The tubes containing the melted agar are placed in a 44.5 ± 0.2°C water bath for 10 minutes to stabilize the temperature.

- 1.0 ml of the E. coli C culture and 5 ml of the water sample are added to each tube of melted MNA. Contents of each tube are gently mixed and poured into separate, labeled Petri dishes (4 plates for a total of 20 ml for each water sample). The Petri dishes are covered, the agar is allowed to solidify, the plates are inverted and incubated at 35 ± 0.5°C. Plaques are counted after 6 hours of incubation. The number of coliphages in the sample is calculated as follows: total plaque forming units (pfu) on the 4 plates (20 ml) x 5 = pfu/100 ml.

- The sensitivity of this procedure is ≥5 pfu/100 ml of sample, based on plating 4 x 5 aliquots of the water sample.

The standard ARCAT procedure was modified for these studies to determine pfu/100 ml of natural water sample by plating 20 x 5 ml water samples. This modification was necessary to increase the sensitivity of the procedure to 1 pfu/100 ml of sample for evaluation of the various concentration techniques.

SECTION 5

EXPERIMENTAL PROCEDURES

FEASIBILITY STUDIES

Concentration techniques with potential for increasing the sensitivity of the ARCAT test to the level of 1 bacteriophage/100 ml of water were selected following a literature search. The techniques evaluated in feasibility studies are listed below:

- Positively charged microporous filters
- Negatively charged microporous filters
- Osmotic filtration
- Controlled lytic bursts
- Adsorption on inorganic salts
- Polymer two-phase separation
- Magnetic filtration
- Electrophoresis

Volumes of water samples used for the feasibility studies varied depending on the concentration technique to be evaluated. Aliquots of 100 to 300 ml were concentrated by osmotic filtration as determined by the dialysis bag size. Lytic bursts were performed using 100 or 200 ml aliquots to achieve the required sensitivity. Adsorption on inorganic salts and filtration permitted concentration of larger volumes (400 to 1,000 ml). Polymer two-phase separations were performed with 250 ml separatory funnels which dictated that 100 ml of sample be used. Magnetic filtration was performed using 100 to 200 ml sample volumes.

Positively Charged Filters

The positively charged filters evaluated during this study were the Zeta Plus filters manufactured by AMF CUNO. Two porosity sizes of these filters were evaluated, the 50S filters (0.75 µm) and the 70C filters (0.2 µm). A thinner filter, the Zeta Plus B0204-1MDS, with porosity near 1 µm was also briefly examined. Water samples tested were taken from the raw water intake from the Occoquan Reservoir and the settled water before chlorination from the Fairfax

County Water Treatment Plant. The pH of the water samples was adjusted from the natural values to 5.0, 6.0 and 7.5 for adsorption of the coliphages onto the filters. In general, 400 ml of the raw water sample at the specified pH were filtered through the Zeta Plus filter and the adsorbed bacteriophages were eluted by washing twice with 10 ml volumes of 3% beef extract, pH 9.0. The filtrate and eluate were plated for enumeration of bacteriophages in ARCAT.

Negatively Charged Filters

Negatively charged filters evaluated for concentration of bacteriophages were the Cox glass filters (2.0 µm and 0.45 µm) and the Bio-Rad nitrocellulose filters (0.03 µm and 0.2 µm). The concentration method consisted of addition of $MgCl_2$ to each water sample to a final concentration of 0.05 M. The pH of the water sample was adjusted from the natural pH to pH 3.5 or pH 5.0 before filtration. Elution was accomplished with two 10 ml washes of a glycine buffer, pH 11.0. The filtrates and eluates were plated in ARCAT for enumeration of bacteriophages.

Osmotic Filtration

Osmotic filtration using dialysis bags (0.635 cm diameter, 0.0089 cm wall thickness, average pore size 4.8 µm) was performed with raw water. In general, the method included soaking 25.4 cm of dialysis tubing in 10% (w/v) glycerine solution for 10 minutes. One end of the tubing was knotted and the tubing filled with 300 ml raw water at room temperature. The top was sealed and the dialysis bag was placed in a 1 liter beaker filled with 100% Carbowax 6000 for adsorption of the water. The bag was removed from the beaker after 22-24 hours and the remaining liquid in the bag was plated using the ARCAT method.

Lytic Bursts

Amplification of bacteriophages by incubation to allow a lytic burst to occur in the water sample was also evaluated. To perform these tests, 6 g of dry TSB and 20 ml of E. coli C host culture ($O.D._{530}$ of 0.5) were added to 200 ml of the water sample to be tested. The samples were incubated at 35 + 0.5°C. Forty ml samples were removed at 0, 10, 20, 30, 45 and/or 60 minute incubation and monitored by ARCAT. Dilutions were made of the samples taken at 45 and 60 minutes.

Adsorption on Inorganic Salts

Adsorption of bacteriophages on inorganic salts was evaluated using aluminum hydroxide and calcium phosphate-calcium hydroxide. These salts were selected based on reports in the literature which indicated that they were commonly used to concentrate bacteriophages (Wallis and Melnick, 1967; Primrose and Day, 1977). The procedure used was as follows. A Bio-Rad unipore disc prefilter was placed on the filtering apparatus. The prefilter was washed with 500 ml of 0.1% Tween 80 solution followed by 1 liter of distilled water. This procedure prevents virus adsorption to the filter (Wallis et al., 1970). For concentration with calcium salts, 20 ml of a suspension of the salts (0.5 M slurry) were layered onto the filter, and a vacuum applied to form a smooth surface. Test water (400 ml - Raw Occoquan water or 800 ml settled Occoquan water) at pH 7.2 was filtered through the salts layer. The calcium salts were

resuspended in 10 ml of a 0.8 M Na_3PO_4 solution (pH 7.2) for bacteriophage elution, centrifuged in a table top centrifuge (Precision Scientific, Vari-Hi-Speed Centricone) at 2500 rpm for 1 minute. The supernatant fraction was decanted for assay of bacteriophages by ARCAT.

For concentration using aluminum hydroxide, 0.5 g of $Al(OH)_3$ was added to 1 liter of water sample and agitated for one hour. The solution was filtered through a Gelman filter (GN6, 0.45 μm). The aluminum hydroxide was resuspended in 0.15 M NaCl to elute adsorbed bacteriophages. The eluate was plated in ARCAT for determination of bacteriophages. Samples from the filtration of the test waters through the salts were also plated in ARCAT to determine the efficiency of bacteriophage adsorption onto the salts.

Polymer Two-Phase Separation

Two-phase polymer separation (Sobsey, 1976) was tested as a method for bacteriophage concentration. Carbowax PEG 6000 and dextran sulfate (sodium salt), molecular weights of 6000-7000 and 20,000 respectively, were used. Sodium chloride (0.1 M) or potassium chloride (0.01 to 0.1 M) was used to enhance separation. For the separation Carbowax (7% or 10%), dextran sulfate (1%, 2% or 5%) and the sodium or potassium salts at the above concentrations were added to 50 or 100 ml water samples. The solutions were incubated at 9°C in separatory funnels for 18-24 hours. Samples were taken from each phase and plated for bacteriophage determinations in ARCAT.

Magnetic Filtration

The apparatus used for the magnetic filtration method of concentration (Rao, 1968) is presented in Figure 1. In this procedure, 50 mg of 20 mesh iron ore magnetite were added to 100 ml of water sample. The addition of calcium chloride (161 ppm) or aluminum sulfate (161 ppm) to the water was also investigated. The solution was then agitated on an electric shaker for 20 minutes and poured through the magnetic filter with the magnetic field activated. The 14,092 Gauss magnet on the Varian A60 NMR was used to supply the magnetic field. The filtrate was collected in the recovery flask and plated for detection of pfu in ARCAT. The magnetite was eluted with two 10 ml aliquots of 3% beef extract, pH 9.0. The eluate was plated in ARCAT and compared to pfu detected in the original sample by ARCAT.

Electrophoresis

An electrophoretic cell was constructed to evaluate electrophoresis as a method for concentration of coliphages. Electrophoretic cells are filtering devices in which a direct current electrical field is applied across the filtering surface. A schematic of the cell is presented in Figure 2.

As illustrated in Figure 3, the sample is fed into the cell by gravity flow (1 ml/min) and an electrical potential (0 to 30 volts D.C.) is applied across the cell by a Fisher Variac, model 116 (Fisher Scientific). Application of the electrical potential encourages bacteriophage migration through the filter and enhances adsorption to the membrane (Bier et al, 1967). Beef extract (3%) was used to elute the bacteriophages adsorbed to the membrane. The eluate was assayed by ARCAT.

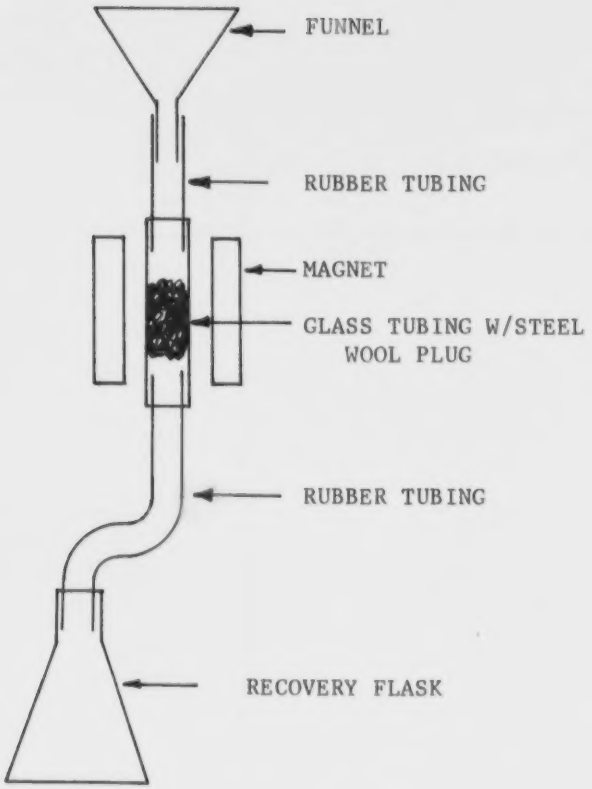


Figure 1. Magnetic Filtration Apparatus

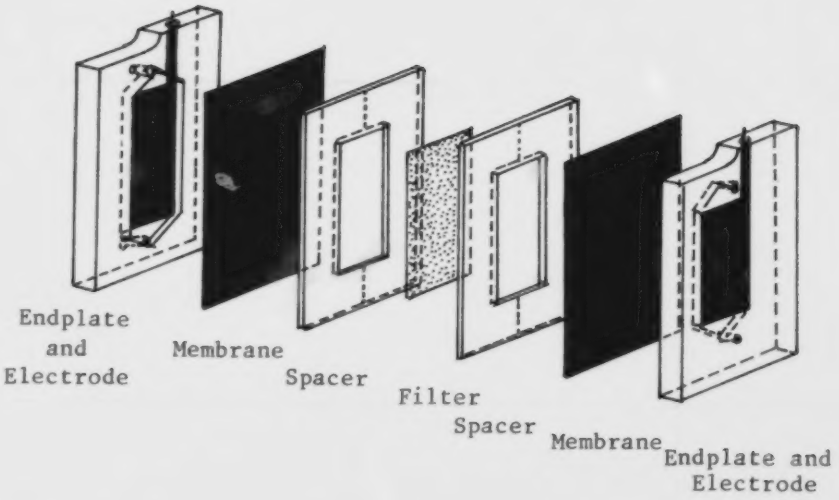
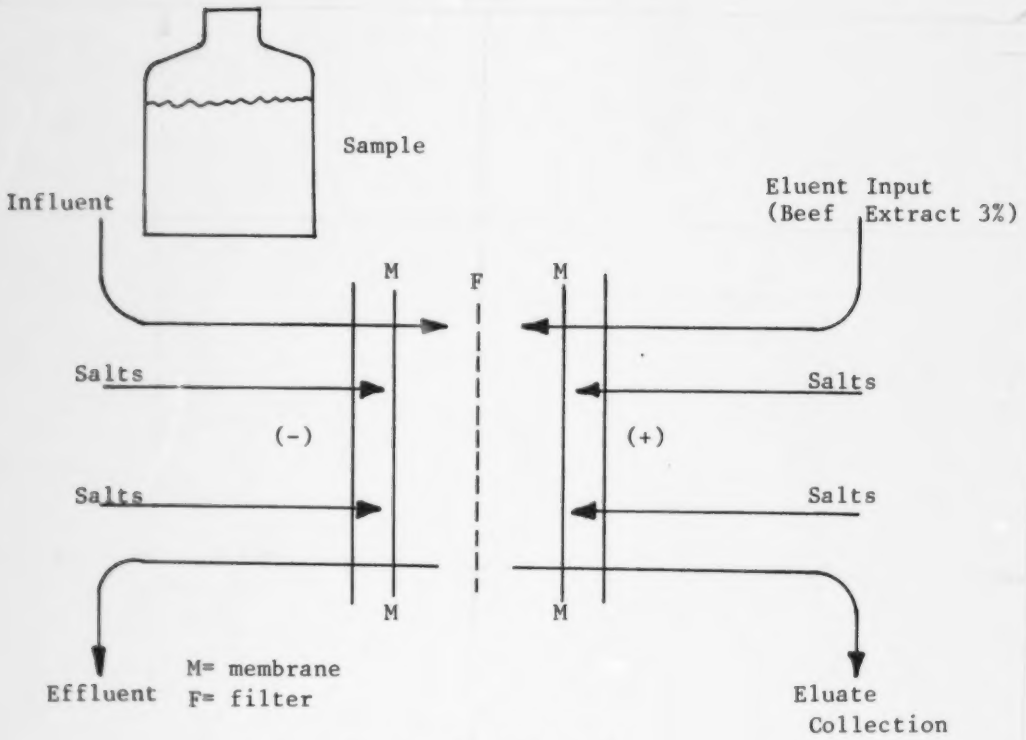
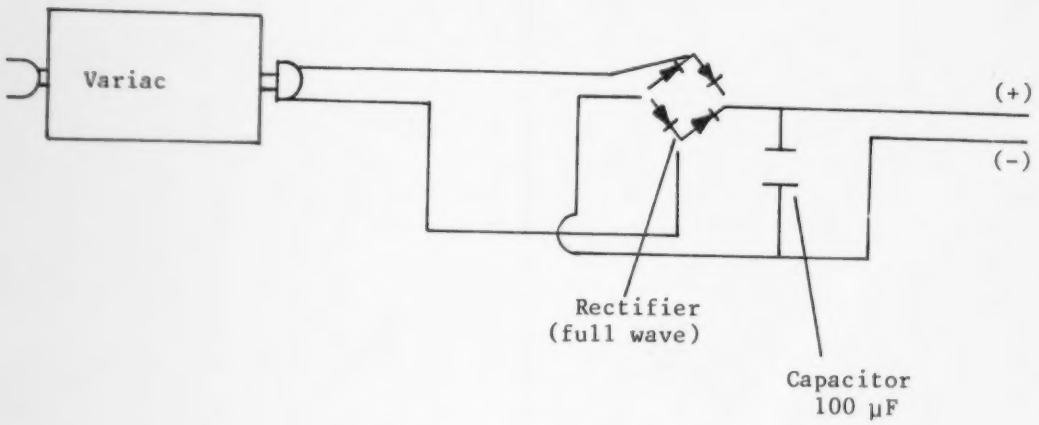


Figure 2. Electrophoretic Cell



Electrophoretic Cell



Power Supply

Figure 3. Electrophoretic Cell and Power Supply

The electrophoretic cell was assembled and prepared for use by flushing with sterile saline (0.1%). The volume of water (500 ml) to be filtered was placed in a sterile container above the electrophoretic cell and attached to the influent hose. A 100 ml aliquot of the same water sample was plated in ARCAT. The cell leaked badly on the first attempt at passing 500 ml of water through the system. The endplates were removed and the cell was reassembled in an attempt to seal the system. Several attempts were made to "filter" water samples but attempts to seal the system were unsuccessful. Since other methods tested in the feasibility studies were ready for optimization, electrophoresis was abandoned in favor of the more promising techniques.

OPTIMIZATION STUDIES

Positive Filters

In general, the positive filter method consisted of filtration of 500 ml of dilute stock bacteriophage. Filtrations were performed with an electric vacuum pump (115 volts 2.0 amps, 1/8 horsepower) that provided suction to 15 mm of mercury. Samples were filtered through the 50S, 60S, 70C, or B0204-1MDS positively charged filters with no manipulation or with manipulation by addition of salt or pH adjustment. A 40 ml aliquot of each filtrate was plated in ARCAT to determine adsorption of bacteriophages. Bacteriophages were eluted from the filters with two 10 ml aliquots of sterile eluent. The eluates were plated in ARCAT.

Phase I - Optimize Adsorption - Optimization of bacteriophage adsorption onto positively charged filters was accomplished by adjusting seeded water samples to pH values ranging from 6.0 to 7.8 prior to filtration. Adsorption characteristics of bacteriophages from a variety of water sources onto the positive filters were determined.

Phase II - Optimize Eluent and Elution Procedures. Three eluents at various pH values and concentrations were evaluated for their ability to remove the bacteriophage from the filter. Glycine, at molarities ranging from 0.05 to 0.001 and at pH values of 9.0 and 11.0, was screened as an eluent. Beef extract was evaluated as 3% beef extract, pH 9.0; 3% beef extract + antifoam agent, pH 9.0; 1:1 8% beef extract and 1 M NaCl, pH 11.0; and 3% beef extract + 0.5 M NaCl, pH 9.0. Trypticase soy broth (3%) was evaluated at pH values ranging from 8.0 to 11.0.

Phase III - Compare Positively Charged Filters. This study examined the 50S, 60S, 70C and B0204-1MDS positively charged filters for adsorption and elution of bacteriophages utilizing the optimal adsorption pH for filtration and trypticase soy broth for elution of bacteriophages.

Negative Filters

The general procedure for concentration of bacteriophages with negatively charged filters consisted of filtration of 500 ml of dilute, seeded water samples at natural pH and at adjusted pH values. Filtration was accomplished with an electric vacuum pump (115 volts 2.0 amps, 1/8 horsepower) that provided suction to 15 mm of mercury. A 40 ml aliquot of each filtrate was plated in ARCAT to

determine adsorption of bacteriophages. Bacteriophages were eluted from the filters with two 10 ml aliquots of sterile eluent. The eluates were plated in ARCAT.

Phase I - Optimize Adsorption. The Cox 0.45 μm negatively charged filter was evaluted for its ability to adsorb bacteriophages from aqueous solutions. The optimum pH for adsorption of bacteriophages to the negative filters was determined by filtration of seeded samples at pH values ranging from 3.5 to 8.6.

Phase II - Optimize eluent and eluate procedure. Glycine (0.01 M) and trypticase soy broth (3%) were evaluated as eluents. The pH values examined ranged from 8.0 to 11.0.

Lytic Bursts

One-Step Growth Experiment--

The experiment used to determine the lytic burst size and burst time for coliphages ΦX174 and T3 was the one-step growth experiment, developed by Ellis and Delbruck (1939) and described by Adams (1959). In this experiment, 0.9 ml of the E. coli C host bacterium (thawed, frozen host) in TSB (3%) was mixed with 0.1 ml of a 10^8 pfu/ml coliphage stock, either ΦX174 or T3, to give a multiplicity of infection (ratio of bacteriophage to host cell) of 0.06 to 0.1. At these multiplicity of infection (m.o.i.) levels, each E. coli C cell has no more than one coliphage adsorbed onto it. Therefore, each pfu scored was the result of one bacterium infected with one coliphage. The adsorption process was allowed to proceed for 15 minutes at a temperature of 35 ± 0.5°C. At the end of 15 minutes, 0.1 ml of the 1 ml adsorption mixture was diluted by a factor of 2×10^5 into a test tube containing 0.9 ml TSB (first growth tube). A further 100-fold dilution was made from the first growth tube into a second growth tube to give a final dilution factor of 2×10^7. The growth tubes were incubated at 35 ± 0.5°C. Duplicate 0.1 ml samples were taken from the first growth tube at 18, 25, 35 and 40 minutes. Duplicate 0.1 ml samples were taken from the second growth tube at 35, 40, 45, 50, 55 and 60 minutes. Each sample was mixed with 2.5 ml soft modified nutrient agar and 0.5 ml fresh E. coli C host and poured over solidified modified nutrient agar in sterile Petri dishes. The dishes were incubated for 6 hours at 35 ± 0.5°C and subsequently scored for pfu.

Single Burst Experiment--

The single burst experiment, as developed by Ellis and Delbruck (1939) and described by Adams (1959), was used to determine the average burst size in seeded natural water samples using the burst time calculated in the one-step growth experiment. In these experiments, 0.9 ml of 10^9 cfu E. coli C/ml was mixed with sterile natural water samples seeded with either bacteriophage ΦX174 or T3 (0.1 ml of a 10^8 pfu/ml stock) to give a m.o.i. of 0.01 to 0.1. The 1 ml mixture was incubated at 35 ± 0.5°C for 15 minutes to allow adsorption to occur. At the end of the adsorption period, the mixture was diluted by a factor of 4×10^7 with TSB. Aliquots of 0.5 ml were taken from the diluted mixture and pipetted into sterile, empty test tubes (10-12 tubes), prewarmed in a 35 ± 0.5°C water bath. These tubes were incubated at 35 ± 0.5°C for 45 minutes for T3 and 60 minutes for ΦX174. At the end of this time, the contents of each tube were mixed with 2.5 ml soft

modified nutrient agar and 0.5 ml of E. coli C host and poured over solidified modified nutrient agar. The plates were incubated at 35 ± 0.5°C for 6 hours and the plaques counted.

Laboratory Amplification of Samples Containing a Single Bacteriophage Type--

The bacteriophage stock (either ΦX174 or T3) was diluted with TSB to ~0.3 pfu/ml. Two ml of E. coli C were added to 100 ml of bacteriophage-TSB to yield 1×10^7 cfu E. coli C/ml (a m.o.i. of 3×10^{-8} to 6×10^{-8}). The bacteriophage-TSB-E. coli C mixture was then incubated for a predetermined time, based on burst time for each bacteriophage as determined in one step growth experiments. At the end of this time period, four 5 ml samples were plated in ARCAT, incubated for six hours and the pfu counted.

Laboratory Amplification of Samples Containing Two Bacteriophage Types --

For amplification of bacteriophage mixtures, ΦX174 and T3 bacteriophage stocks were diluted to ~7 pfu/100 ml and ~2 pfu/100 ml, respectively. Fifty ml of each diluted bacteriophage stock were placed in three flasks. The three flasks thus contained 100 ml of sample with ~3.5 pfu ΦX174 and ~1 pfu T3. The same amplification/plating procedure employed with the single bacteriophage was used in the double bacteriophage amplification. Samples were plated at 40, 45 and 50 minutes.

Amplification of Raw Water Samples--

For amplification of raw water samples, 100 ml of the water were pipetted into sterile flasks and prewarmed to 35 ± 0.5°C. Two ml of E. coli C (1×10^9 cfu/ml) were added to each flask to give 1×10^7 cfu E. coli C/ml per flask. Three grams of dry TSB were added to the flasks as a nutrient source. The flasks were incubated at 35 ± 0.5°C for 50 minutes. Four 5 ml samples were taken at 30, 35, 40, 45 and 50 minutes. The 30 minutes sample was taken from flask 1, the 35 minutes sample from flask 2, etc. Therefore each flask was sampled only once. The samples were mixed with modified nutrient agar, plated and incubated at 35 ± 0.5°C for 6 hours. At 6 hours, the pfu were scored.

Optimization of Incubation Time for Natural Water Samples--

Experiments were performed in the following manner. For each experiment, 100 ml of the natural water sample were pipetted into each of 3 sterile flasks. Three g of dry TSB were added to each flask and the flasks warmed for 15 minutes at 35 ± 0.5°C. E. coli C was thawed at 44.5 ± 0.2°C for 15 minutes and 20 ml of this stock host culture was added to each flask to give 1×10^8 cfu E. coli C/ml per flask. The flasks were shaken and incubated at 35 ± 0.5°C. Four 5 ml samples were taken at 50, 70 and 80 minutes. Each flask was sampled only once, so that samples taken at 50 minutes were drawn from flask 1, samples at 70 minutes from flask 2 and 80 minute samples from flask 3. The samples were plated in ARCAT, incubated for 6 hours and the pfu scored. A regular ARCAT was also performed on the same water sample for comparison of ARCAT counts and amplification counts. This sampling method resulted in variation in the types of pfu present in each sample as well as in total counts. Bacteriophages present at very low levels (i.e. 1-5 pfu/100 ml) in water samples can result in different exact numbers and

types of bacteriophage present in individual flasks. However, the one-time sample per flask most accurately duplicates the procedure that would be used for an amplification by a water quality laboratory.

VERIFICATION STUDIES

In the verification studies, the recovery of bacteriophages using concentration with the Zeta Plus 60S filter was compared with the results obtained by plating 100 ml of sample in the regular ARCAT method. All waters used in the verification studies were natural waters or waters from water treatment plants in the Washington, D.C. area.

For the regular ARCAT method, 100 ml of water sample were plated. Five ml aliquots of the sample were combined with 5 ml of modified nutrient agar and 1 ml of E. coli C host culture (frozen host thawed to 44.5 $\pm$ 0.2°C prior to use). Each mixture was plated (20 plates total) and incubated at 35°C $\pm$ 0.5°C for 6 hours. The plates were scored for pfu at 6 hours.

Prior to filtration through the Zeta Plus 60S filters the samples were adjusted to the optimum adsorption pH (6.0) as determined in the optimization studies. Volumes of water filtered ranged from 100 to 1000 ml. The adsorbed bacteriophages were eluted with 10 or 20 ml of TSB adjusted to pH 8.5. The eluates were plated in 5 ml volumes as in the regular ARCAT method. Samples were concentrated by factors of 5, 10, 25 or 50.

During the verification studies, the bacteriophage pfu/100 ml detected were compared with fecal and total coliform cfu/100 ml. The fecal and total coliform cfu/100 ml were determined by Standard Methods membrane filter procedures as follows.

For determining total coliforms, M-Endo Agar LES (BBL) (51 g/l) was rehydrated in distilled water containing 20 ml 95% ethanol and brought to a boil on a stirring, hot plate. The agar was cooled to 45°C and 4.5 ml volumes pipetted into Falcon #1007, 60 x 15 mm Petri dishes. The Millipore filter apparatus was assembled, covered with aluminum foil and sterilized by autoclave for 15 minutes at 15 psig. Sterile Gelman GN-6 0.45 um grid filters were transferred to the cooled Millipore apparatus using alcohol/flame sterilized forceps. Assay of water samples of less than 20 ml volume required application of two 10 ml volumes of sterile dilution water to the filter before sample filtration and following sample filtration. Dilution water was prepared by dissolving 34 g of potassium dihydrogen phosphate in 500 ml distilled water. The pH was adjusted to 7.2 with 1 N NaOH and the total volume was adjusted to one liter with distilled water (solution 1). Solution two was prepared by addition of 50 g of $MgSO_4.7H_2O$ to one liter of distilled water. A 1.25 ml aliquot of solution two were combined with distilled water to make one liter of dilution water. Ten ml volumes of dilution water were pipetted into tubes, which were capped and autoclaved. Aliquots of the sterile dilution water were used for dilutions.

Sample volumes varied according to the source of the sample and ranged from 0.001 ml to 100 ml filtered. Two, three or four dilutions were used for each sample. Following sample filtration, filters were removed with sterilized forceps and placed, grid side up on the agar plates. The plates

were inverted and incubated at 35°C ± 0.5°C for 24 hours. All organisms that produced a colony that showed a golden-green metallic sheen within 24 hours were considered members of the coliform group. These colonies were counted and coliform density in terms of total coliforms/100 ml calculated by the following equation:

$$\text{(Total) coliform colonies/100 ml} = \frac{\text{coliform colonies counted} \times 100}{\text{ml sample filtered}}$$

Total coliforms were verified by picking 10% of the positive colonies from the total coliform plates and introducing each into sterile lauryl tryptose broth (LST) and brilliant green bile (BGB) tubes containing Durham tubes for detection of gas production due to fermentation of lactose. Tubes were incubated at 35°C ± 0.5°C for 24-48 hours. Growth in the broth tubes and gas production in the germ tubes confirmed a positive total coliform colony. Confirmations showing LST (+) and BGB (-) were subcultured from LST into fresh BGB for detection of gas production. Where confiration did not occur, the original total coliform computation was adjusted by the following equation:

$$\text{total coliform colonies/100 ml} - \frac{\text{negative colonies in verification}}{\text{number of colonies subjected to verification}} \times 100 = \text{adjusted total coliforms/100 ml}$$

For the fecal coliform determination, M-FC agar (52 g, Difco) was dissolved in one liter of distilled water and brought to a boil on a stirring hot plate. When boiling was reached, 10 ml of 1% rosolic acid in 0.2 N NaOH per liter of agar was added and boiled for one minute before cooling to 45°C. The cooled agar was pipetted in 4.5 ml volumes into Falcon #1006 50 x 9 mm locking Petri dishes. The plates were inverted and stored at 5°C for no more than 4 days. The Millpore filter apparatus was assembled, covered with aluminum foil and sterilized by autoclave for 15 minutes at 15 psig. Sterile Gelman GN-6 0.45 m grid filters were transferred to the cooled Millipore apparatus using alcohol/flame sterilized forceps. Filtration was accomplished as described for the Standard Total Coliform Membrane Filter Procedure. Sample volumes varied according to the source of the sample and ranged from 0.001 ml to 100 ml filtered. Two, three or four dilutions were used for each sample. Following sample filtration, filters were removed with alcohol/flame sterilized forceps and placed, grid side up, on the agar plates. The prepared plates were placed in waterproof plastic bags for protection during submersion in the water bath for the 24 hour incubation at 44.5 ± 0.2°C. The dishes were anchored below the water surface during incubation to maintain critical temperature requirements. The blue colonies produced by fecal coliform bacteria were counted and the fecal coliform density was computed by the following equation:

$$\text{(fecal) coliform colonies/100 ml} = \frac{\text{coliform colonies counted} \times 100}{\text{ml sample filtered}}$$

The bacteriophages were quantitated using the regular ARCAT method. Fecal and total coliforms and the standard plate count bacteria were quantitated by the standard procedures described in the previous section. The

total bacteria present in the samples were determined by the standard plate count using the following procedure.

For most potable water samples, plating of 1 ml and 0.1 ml of undiluted water sample and 1 ml of water sample diluted 1:100 with sterile dilution water was suitable. Tubes of sterile, solid plate count agar were melted by exposure to flowing steam. The melted medium tubes were coded and placed in a water bath between 44°C and 46°C. As the medium from each tube was poured into the plate, the melted medium was thoroughly mixed with the test portions in the Petri dish by rotating the plate in one direction and then in the opposite direction. The plates were allowed to solidify on a level surface, inverted and placed in an incubator at 35 $\pm$ 0.5°C for 48 $\pm$ 3 hours. All colonies on plates containing 30 to 300 colonies were counted. The total number of colonies or the average number (for duplicate plates of the same dilution) per plate were multiplied by the reciprocal of the dilution to compute the Standard Plate Count.

EFFECTS OF PHYSICAL AND CHEMICAL TREATMENT ON BACTERIOPHAGES AND BACTERIA

Physical Treatment

To determine the effects of flocculation and filtration on bacteriophages and bacteria, samples were collected from the Potomac Plant on River Road in Potomac, Maryland. The Potomac plant does not add chlorine until after filtration has taken place. For flocculation, ferric chloride at 9 to 40 mg/L and lime at 12 to 24 mg/L are added. Retention time is 28 minutes in the floc basin and 2.25 hours in the sediment basin (based on 160 MGD). Filtration is accomplished by 16 units of 16 inch anthrafill, 12 inch gravel and 10 inch sand with a filter cap of 2.7 G per foot per minute (160 MGD). Raw samples were drawn from the plant influent tap (raw Potomac River water) located in the laboratory of the facility. The settled samples were taken from the laboratory influent tap to the filtration tank as the waters leave the settling tank prior to entry into the filtration tank. The filtered samples were drawn from the filtration tank effluent tap, prior to chlorination. A schematic drawing of the treatment facility and points of sample collection is presented in Figure 4. The pH, temperature and turbidity of the raw, settled and filtered samples were determined in addition to the bacteriophages and total and fecal coliforms.

Disinfection Study: Enrichment Culture

Bacterial and bacteriophage levels in natural waters in the Washington, D.C. area during the summer and fall of 1980 were very low due to drought conditions. To provide natural-like bacteria and bacteriophages, natural water samples were introduced into the biodisc to produce an enrichment culture. The biodisc is a rotating biological contactor made up of a series of four aeration chambers. In this instance, the biodisc was used to enhance the growth of microorganisms found in the natural water samples used throughout this project (i.e., Potomac River, Accotink Creek, Occoquan Reservoir). The stage one aeration chamber was supplemented with sterile TSB (25 ml of a 10X TSB solution) once a week. Samples were drawn from the outflow of the first aeration chamber.

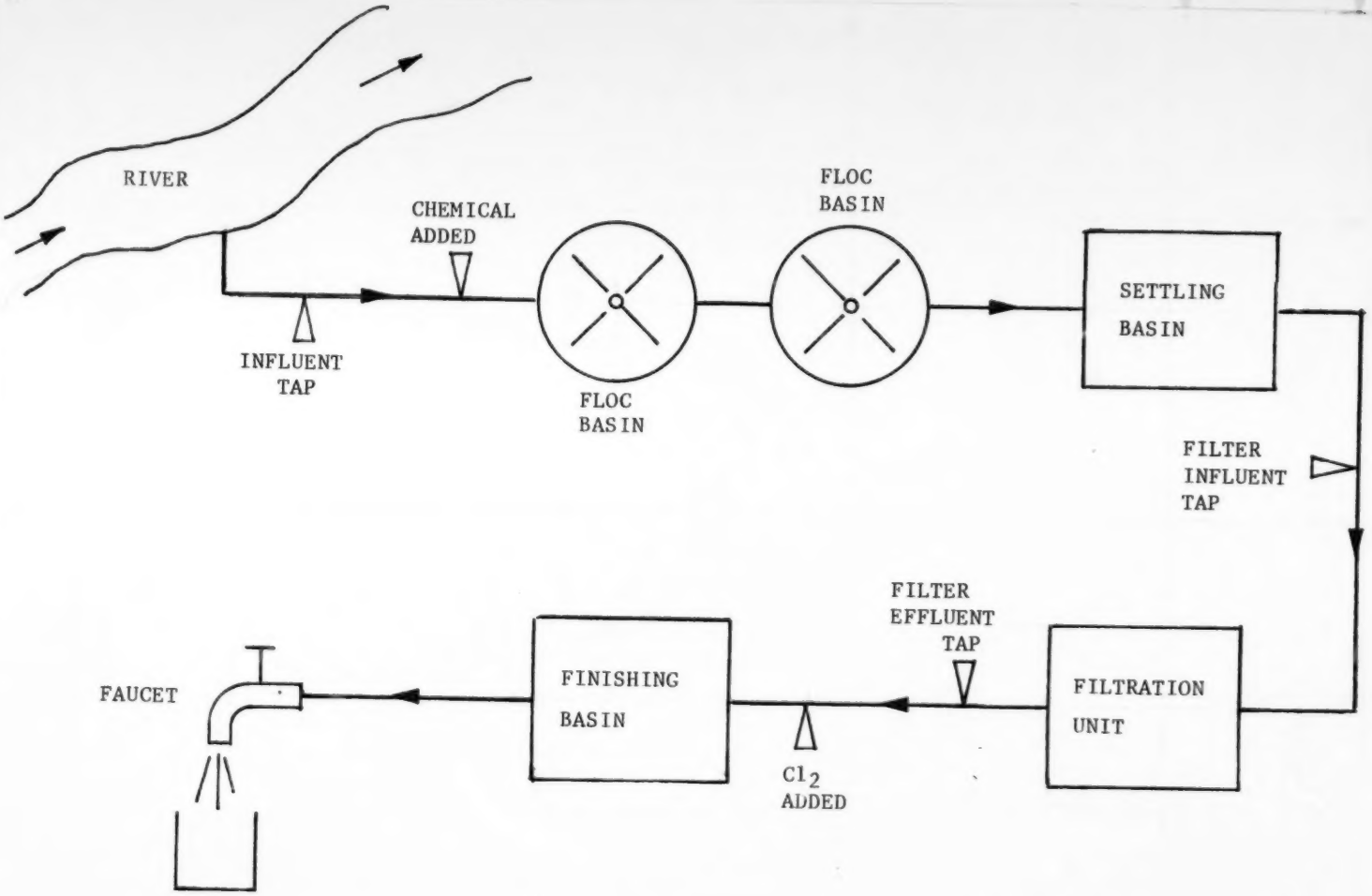


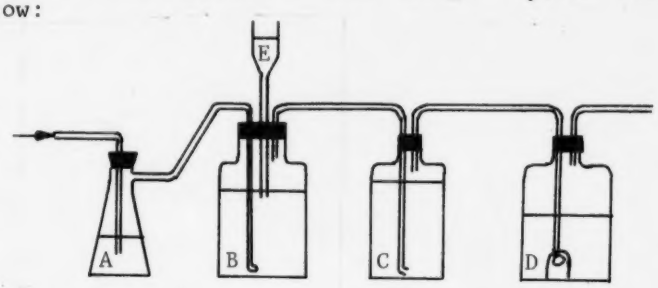
Figure 4. Potomac Filtration Plant

Ultraviolet Light

Samples (50 ml or 100 ml) from the enrichment culture were diluted with sterile raw water and exposed to ultraviolet light from a shortwave UV lamp (254 nm) in 15 x 150 mm Petri dishes (liquid depth of 0.42 or 0.80 cm) at a distance of 54.6 cm from the light source. The distance (54.6 cm) from the ultraviolet source was selected as a practical distance for supplying the disinfection. Two sample depths were monitored to determine the effect of depth on disinfection. Following exposure, samples (100 ml) were monitored for bacteriophages using the ARCAT method and for fecal and/or total coliforms using Standard Methods.

Chlorine Dioxide

The effect of chlorine dioxide as a disinfectant was studied with respect to bacteria and bacteriophages present in the biodisc enrichment culture at Atlantic Research. The chlorine dioxide was produced in the laboratory according to Standard Methods (14th Edition), 411A using a system similar to the one illustrated below:



The gas generating-adsorbing system was prepared by connecting aspirator flask A (500 ml capacity) by means of rubber tubing to a source of compressed air. Air was filtered by bubbling it through a layer of 300 ml of distilled water in flask A and then passed over and down through a glass tube to within 5 mm of the bottom of the 1-liter gas-generating bottle B. The evolved gas was conducted via glass tubing through a scrubber bottle C containing saturated sodium chlorite solution and finally via glass tubing into a 2-liter pyrex collecting bottle D, where the gas was absorbed in 1,500 ml distilled water. An air outlet was provided on bottle D for escape of the moving air. The system was located in a fume hood. Ten grams of sodium chlorite were dissolved in 750 ml distilled water and placed in bottle B. Two ml of concentrated H_2SO_4 were added to 18 ml distilled water and mixed. This solution was transferred to cylinder E. Flask A was connected to bottle B and bottle B to bottles C and D. A smooth current of air was passed through the system. Five ml increments of sulfuric acid from cylinder E were introduced into bottle B at 5 minute intervals. The air flow was continued for 30 minutes after the last portion of acid had been added. The yellow stock solution was stored in a dark refrigerator. The concentration of the chlorine dioxide was determined by titration with standard 0.01 N or 0.025 N sodium thiosulfate titrant in the presence of KI, acid and starch indicator. Daily standardizations were performed and the amount of chlorine dioxide required for the desired chlorine levels was added to 500 ml water samples as determined by the formula:

$$\text{mg/L Cl} = \frac{(A + B) \times N \times 35.450}{\text{ml sample}}$$

A = ml used for titration of sample
B = ml used for titration of blank
N = normality of $Na_2S_2O_3$

A sample from the ARC biodisc enrichment culture was diluted 1:50, 1:200, or 1:300 in sterile distilled or sterile tap water at natural pH of approximately 7.0. Chlorine in the form of chlorine dioxide was added to 500 ml volumes of the dilute sample at levels of 1.0, 2.5, 5.0 and 10.0 ppm. After fifteen minutes, the residual free chlorine levels were determined using the Hach High Range Chlorine Test Kit model CN-21P and the Free and Total Chlorine Test Kit (0-3 mg/L) model CN-6, and the chlorine was neutralized using 10% sodium thiosulfate. Samples were then evaluated for bacteriophage by the ARCAT method, for fecal and total coliforms according to Standard Methods, and for total bacteria by Standard Methods.

Ozone

Ozone was examined as a method for disinfection of water. The apparatus used for ozone generation and its provision to the water sample is illustrated in Figure 5. The oxygen was converted to ozone in the quartz tube by exposure to ultraviolet light (wavelength of 254 mm). Ozone produced was then passed through the glass tubing and bubbled out of the fritted glass end into a 500 ml water sample in a glass cylinder. A second glass tube connected to a vacuum aspirator drew off excess gas from the head space in the cylinder.

Water samples were taken from the ARC biodisc enrichment culture, pH 7.0 - 7.2, daily and diluted either 1:100 or 1:200 using sterile tap water as a diluent. These samples were exposed to ozone for periods of 0, 1, 2.5, 5.0 and 10 minutes. The oxygen flow rate used for ozone generation was measured because the ozone actually generated could not be measured during ozonation. Residual ozone levels in the water samples were evaluated immediately following the specified ozone exposure time using the Hach Ozone Test Kit model OZ-2, for levels of 0-2 mg/L. The samples were monitored for bacteriophages by the ARCAT method, total and fecal coliforms by Standard Methods and total bacteria by Standard Methods.

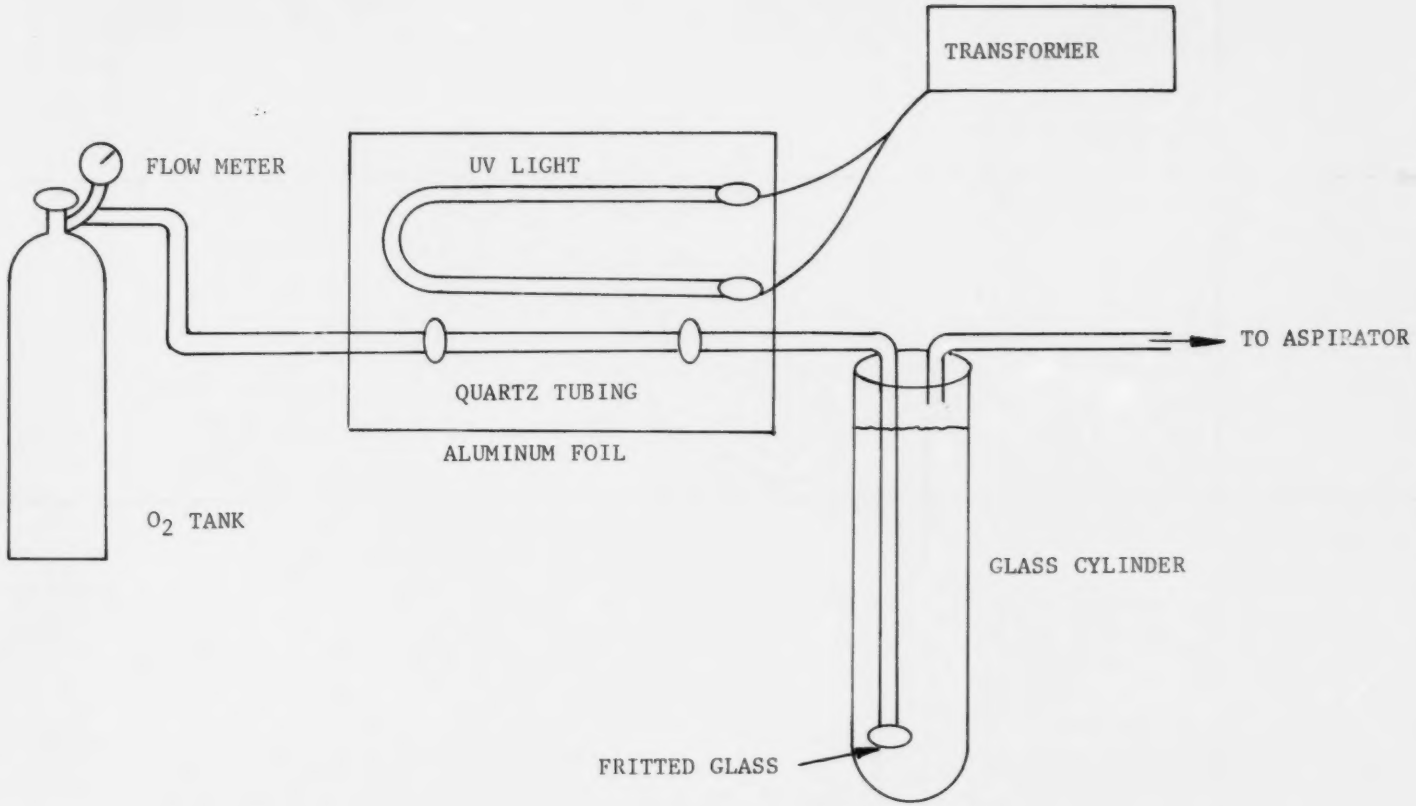


Figure 5. Apparatus for Ozonation of Water

SECTION 6

RESULTS AND DISCUSSION

FEASIBILITY STUDIES

Positive Filtration

The results of the Zeta Plus positively charged filter studies are presented in Table 1. Adsorption of bacteriophages (pfu observed in the filtrates) appeared to be incomplete following filtration of several of the water samples through the 70C positively charged filter. Recoveries appeared to be best when raw water samples were adjusted to pH 6.0 rather than the natural water pH of 7.0-7.3. Filtration of 400 ml of water through the 70C filter required 30 minutes for completion.

Adsorption of bacteriophages was good with the 50S filter as demonstrated by the presence of pfu in only one of three filtrate samples assayed. Recoveries from natural waters filtered at pH values of 6.0, 7.5 and natural pH (6.7-6.8) did not demonstrate significant differences. Recoveries from settled waters were best when the pH of the sample was adjusted to pH 6.0 prior to filtration. Only 20 minutes were required for filtration of 400 ml of raw water using the 50S filter.

The third filter, B0204-1MDS, allowed filtration of 400 ml raw water samples in less than 10 minutes. Recoveries were good when the water sample was adjusted to pH 6.0 prior to filtration. Even though no bacteriophage were detected in 100 ml of original water sample on two sample days, the concentration technique demonstrated recovery of 1 bacteriophage (3/3) and 3 bacteriophages (3/4). Filtration of 1,000 ml on sample day 3/4 demonstrated bacteriophage in the filtrate, i.e. incomplete adsorption. The bacteriophage detected in the filtrate account for only 3% of the bacteriophages applied to the filter. Forty-eight percent of the bacteriophages applied to the filter were recovered in the eluate and the remaining bacteriophages may have been undetected in the filtrate, retained on the filter or inactivated in some way.

Negative Filtration

The results of the negatively charged filter studies are presented in Table 2. Filtration was accomplished in less than 5 minutes with the Cox 2.0 µm filter but bacteriophages were detected in the filtrates. Recoveries from raw water samples appeared to be best when samples were adjusted to pH 3.5.

The Bio-Rad 0.2 µm filter required in excess of 2 hours for filtration of 400 ml of raw water. Bacteriophage retention was very good, with only 1 pfu

TABLE 1. BACTERIOPHAGE CONCENTRATION WITH ZETA PLUS POSITIVE FILTERS

Date	Filter Type	Water Sample	Filtration pH	ø/100 ml[1] ARCAT	Volume Filtered (ml)	Eluate Volume (ml)	Concentration Factor	ø Recovered	2 ø Recovered[2]	ø in Filtrate
2/28	70C	Raw	7.0	10	400	20	20	26	65.0	1
		Raw	6.0	10	400	20	20	21	52.5	3
		Settled	6.8	<1	800	20	40	4	*	0
		Settled	6.0	<1	800	20	40	4	*	-
2/29	70C	Raw	7.3	20	400	23	17	22	27.5	2
		Raw	6.0	20	400	21	19	43	54.0	-
		Settled	7.2	<1	800	23	35	10	*	0
		Settled	6.0	<1	800	23	35	10	*	-
2/15	50S	Raw	6.8	34	300	20	15	74	73.0	0
		Raw	6.0	34	400	20	20	84	62.0	-
		Raw	7.5	34	300	20	15	64	63.0	-
		Settled	6.8	3	500	20	25	3	20.0	-
		Settled	6.0	3	832	20	42	13	52.0	-
2/14	50S	Raw	6.8	36	400	20	20	92	64.0	1
		Raw	6.1	36	400	20	20	63	44.0	-
		Raw	7.5	36	400	30	30	89	62.0	-
		Settled	6.7	2	800	20	40	2	13.0	-
		Settled	6.0	2	800	20	40	7	44.0	-
		Settled	7.5	2	500	20	25	3	30.0	-
2/20	50S	Raw	6.7	28	400	20	20	57	51.0	0
		Raw	6.0	28	400	20	20	59	53.0	-
		Raw	5.0	28	400	20	20	57	51.0	-
		Settled	6.7	5	800	20	40	8	20.0	-
		Settled	6.0	5	800	20	40	12	30.0	-
		Settled	5.0	5	800	20	40	5	13.0	-
3/3	B0204 1MDS	Raw	7.1	3	400	20	20	5	42.0	0
		Raw	6.0	3	400	20	20	13	108.0	-
		Settled	6.9	<1	800	20	40	1	*	-
3/4	B0204 1MDS	Raw[3]	6.0	6	1000	20	50	29	48.0	2
		Settled	6.0	<1	2000	20	100	3	*	0

The eluent used in these experiments was beef extract, 3%, pH 9.0.

2. Based on comparison of ø recovered and ø in original sample.

* Unable to evaluate; no bacteriophage detected in 100 ml original sample.

1. ø - bacteriophage; 100 ml assayed by ARCAT

3. Double filter - two thicknesses of 1 MDS filter.

- Not evaluated for presence of bacteriophage.

TABLE 2. BACTERIOPHAGE CONCENTRATION WITH NEGATIVE FILTERS

Date	Filter Type	Water Sample	Filtration pH	Ø/100 ml[1] ARCAT	Volume Filtered (ml)	Eluate Volume (ml)	Concentration Factor	Ø Recovered	% Ø Recovered[2]	Ø in Filtrate
2/28	Cox	Settled	6.5	5	600	20	30	1	3.3	0
	M-780	Settled	5.0	5	600	20	30	6	20.0	-
	2.0 µm	Settled	3.5	5	600	20	30	14	47.0	-
		Raw	6.8	14	300	20	15	9	21.0	3
		Raw	5.0	14	300	20	15	6	14.0	-
		Raw	3.5	14	300	20	15	17	40.0	-
2/27	Cox	Settled	6.7	<1	700	20	35	6	*	-
	M-780	Settled	5.0	<1	700	20	35	6	*	-
	2.0 µm	Settled	3.5	<1	700	20	35	3	*	-
		Raw	6.8	15	400	20	20	13	22.0	7
		Raw	5.0	15	400	20	20	9	15.0	-
		Raw	3.5	15	400	20	20	18	30.0	-
3/10	Bio-Rad	Raw	6.8	8	400	20	20	0	0.0	1
	0.2 µm	Raw	3.5	8	400	20	20	1	3.0	-
		Settled	6.6	1	800	20	40	2	25.0	0
		Settled	3.5	1	800	20	40	2	25.0	-
3/11	Bio-Rad	Raw	6.8	5	400	20	20	0	0.0	-
	0.2 µm	Raw	3.5	5	400	20	30	5	25.0	-
		Settled	7.0	1	700	20	35	4	57.0	-
		Settled	3.5	1	700	20	35	5	71.0	-
3/12	Cox	Raw	7.1	1	400	20	20	2	50.0	0
	0.45 µm	Raw	3.5	1	400	20	20	5	125.0	-
		Settled	6.8	<1	800	20	35	1	*	-
		Settled	3.5	<1	800	20	35	1	*	-
3/11	Cox	Raw	7.1	3	400	20	20	0	0	0
	0.45 µm	Settled	6.8	1	700	20	35	5	71.0	0
		Settled	5.0	1	700	20	35	0	0.0	-
		Settled	3.5	1	700	20	35	1	14.0	-

The eluent used in these experiments was 0.05 M Glycine in 1 N NaOH, pH 11.0.
1. Ø - bacteriophage; 100 ml assayed by ARCAT.
2. Based on comparison of Ø recovered and Ø in original sample.
* Unable to evaluate; no Ø detected in 100 ml original sample.
\- Not evaluated for presence of bacteriophage.

detected in the three filtrate samples tested. The best recoveries were obtained from concentration of raw and settled water samples when samples were adjusted to pH 3.5.

Adsorption appeared to be good with the Cox 0.45 µm filter and sample filtration was generally completed in 30 to 60 minutes. No bacteriophages were detected in the three filtrate samples tested. Recoveries were inconsistent as demonstrated by adsorption at pH 7.1 and recovery of 50% and 0%, adsorption at pH 3.5 and recovery of 125% and 14%. As adsorption with the filter was good, it is probable that better elution procedures could result in better bacteriophage recoveries.

Osmotic Filtration

A preliminary osmotic filtration experiment demonstrated concentration of a water sample from 300 ml to 44 ml over a 22 hour period. Bacteriophage recovery in this test was 107%. Subsequent tests indicated that times greater than 8 hours (normal working day) were required to concentrate 100 ml samples of raw water to 50 ml (one half the original volume). Bacteriophage recoveries from these samples were not acceptable, as they ranged from 0 to 17.2%. Settled samples were somewhat larger in volume (300 ml compared to 100 ml) because they were expected to contain fewer bacteriophages. Five of the seven settled water samples went to dryness during overnight dialysis. These bags were rinsed with 10 ml of distilled water and the rinse water was analyzed by ARCAT. As shown in Table 3, recoveries of bacteriophages from these experiments ranged from 0 to 150%. The two settled samples which did not go to dryness gave recoveries of 400% and 25.2%. The low number of bacteriophages present in the settled water samples contributed to wide variations in calculated percent recovery.

Lytic Bursts

Results of the lytic burst studies are presented in Table 4. Amplification of the raw water sample on 3/20 resulted in a 16 fold increase in pfu by 60 minutes. Amplifications of other raw samples were achieved by 60 minutes on sample days 3/25 (2,249 fold increase) and 3/28 (406 fold increase). Settled water samples contained low levels of bacteriophages, and amplification of two bacteriophages per 100 ml to 60 pfu (30 fold increase) by 90 minutes was shown on sampling day 3/26. Amplification was also observed on 3/28 with amplification of 2 pfu/100 ml to 518 pfu (259 fold increase) by the 60 minute sampling time and 32,388 pfu by the 90 minute sampling time. The increase in pfu depends upon the burst size of the bacteriophage(s) present and may range from 5-2000. A 10 fold increase was considered significant and indicated amplification of bacteriophages present in the water sample.

Inorganic Salts

The inorganic salts concentration experimental results are presented in Tables 5 and 6. Although adsorption of bacteriophages onto the calcium salts appeared to be good (no pfu detected in filtrates tested), poor recovery of bacteriophages was achieved from four raw water samples and one settled water sample tested. When aluminum hydroxide was used for concentration, bacteriophages were detected in two of four filtrate samples tested, thus indicating that

TABLE 3. BACTERIOPHAGE CONCENTRATION BY OSMOTIC FILTRATION

Date	Water Sample	Temp. (°C)	Ø /100 ml[1] ARCAT	Original Volume (ml)	Final Volume (ml)	Concentration Factor	Ø Recovered	% Ø Recovered
3/17	Raw	25	15	300	44	6.8	48	106.7
3/26	Settled	25	2	100	0* (10)	10.0	3	150.0
	Settled	25	2	100	0* (10)	10.0	2	100.0
	Settled	25	2	100	0* (10)	10.0	1	50.0
	Settled	25	2	100	0* (10)	10.0	0	0.0
	Settled	25	2	100	0* (10)	10.0	0	0.0
3/27	Raw	25	29	100	0* (14.5)	10.0	3	10.3
	Raw	25	29	100	0* (13)	7.7	5	17.2
	Raw	25	29	100	0* (10)	10.0	1	3.4
	Settled	25	2	300	34.5	8.7	24	400.0
3/31	Raw	9	54	100	8	12.5	19	35.2
	Raw	35	54	100	0* (10)	10.0	3	5.6
	Raw	45	54	100	0* (10)	10.0	1	1.8
	Raw	25	54	100	0* (10)	10.0	6	11.1
	Settled	25	1	300	26	11.5	0	0.0

* Samples went to dryness. Bags rinsed with distilled water and the rinse water (volume indicated in ml) was then plated.

1. Ø - bacteriophage; 100 ml assayed by ARCAT.

TABLE 4. AMPLIFICATION OF BACTERIOPHAGES BY LYTIC BURSTS

Date	Water Sample	Ø/100 ml** ARCAT	Ø/100 ml at various plating times (min)*						
			0	10	20	30	45	60	90
3/20	Raw	87	82.5	100	92.5	132.5	200	1380	-
	Raw + Carbon	40	67.5	115	155	102.5	375	8770	-
	Settled	1	<2.5	<2.5	<2.5	<2.5	13	<2.5	-
3/25	Raw + Carbon	9	30	42.5	35	88	1344	20242	506542
	Settled	<1	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	11
3/26	Settled	2	<2.5	-	-	2.5	-	<2.5	60
3/28	Settled	2	<2.5	-	-	-	-	518	32388
	Raw + Carbon	24	22.5	-	-	32.5	313	9750	-

- Sample was not taken at this time.

* Actual samples plated (8-5 ml samples or 40 ml total) were less than 100 ml; the counts have been adjusted by calculation to pfu/100 ml.

** 100 ml assayed by ARCAT.

TABLE 5. BACTERIOPHAGE CONCENTRATION USING CALCIUM PHOSPHATE AND CALCIUM HYDROXIDE

Date	Water Type	Volume of Slurry (ml)	Filtering Time (min)	Volume Filtered (ml)	Final Volume (ml)	Concentration Factor	Ø/100 ml[1] in ARCAT	Ø Recovered	% Ø Recovered[2]	Ø in Filtrate
3/5	Raw	20	90	400	20	20	9	2	5.6	0
	Raw	10	45	400	20	20	9	2	5.6	0
3/6	Raw	10	64	400	20	20	10	0	0	0
	Raw	5	39	400	20	20	10	1	2.5	0
	Settled	5	35	800	20	40	1	0	0	0

1. Ø - bacteriophage; 100 ml assayed by ARCAT.
2. Based on comparison of Ø recovered and Ø in original sample.

TABLE 6. BACTERIOPHAGE CONCENTRATION USING ALUMINUM HYDROXIDE

Date	Water Type	Eluate pH	Volume Filtered (ml)	Final Volume (ml)	Concentration Factor	Ø/100 ml[1] in ARCAT	Ø Recovered	% Ø Recovered[2]	Ø in Filtrate	% Ø in Filtrate
3/17	Raw	6.0	500	20	25	15	1	1.3	-	-
	Raw	7.0	500	20	25	15	0	0	7	9.3
	Settled	6.0	1000	20	50	2	0	0	0	0
	Settled	6.6	1000	20	50	2	0	-	-	-
3/19	Raw	3.0	400	20	20	33	0	0	-	-
	Raw	7.0	400	20	20	33	1	0.8	4	3.0
	Raw	11.0	400	20	20	33	3	2.3	-	-
	Settled	3.0	800	20	40	2	0	0	-	-
	Settled	7.0	800	20	40	2	0	0	0	0
	Settled	11.0	800	20	40	2	0	0	-	-

1. Ø - bacteriophage; 100 ml assayed by ARCAT.
2. Based on comparison of Ø recovered and Ø in original sample.
- Not evaluated for presence of bacteriophage.

good adsorption was not always obtained. Recovery of bacteriophages from the aluminum hydroxide was poor in all samples tested.

Polymer Two-Phase Separation

The results of concentration tests using polymer two-phase separation are presented in Table 7. Bacteriophage recoveries were consistently above 100%. Concentration of the bacteriophages was not observed in any of the five phases monitored. The high recoveries may have resulted from lytic bursts in the raw water samples caused by the addition of KCl, dextran sulfate and Carbowax.

Magnetic Filtration

The magnetic filtration studies results are presented in Table 8. The addition of calcium chloride (161 ppm) to raw water samples resulted in filtrates containing bacteriophage, thus, not all of the bacteriophage were adsorbed in these tests. Bacteriophage recovery of 37.5% was accomplished in one of eight tests from a raw water sample to which aluminum sulfate (161 ppm) was added. All water samples tested contained very low numbers of bacteriophages per 100 ml, making data evaluation difficult.

Evaluation of Concentration Techniques and Selection of Techniques for Optimization

Seven bacteriophage concentration techniques were evaluated with respect to the following criteria:

- Total time required to perform the technique and analyze results.
- Efficiency of bacteriophage recovery compared to original ARCAT determinations of total bacteriophages present.
- Complexity of the technique.

The concentration techniques are compared in Table 9.

Concentration of bacteriophages utilizing positively and negatively charged filters yielded promising results. The positively charged filters (50S, 70C, 1MDS) gave good adsorption in most cases, but bacteriophage recoveries were erratic. Optimization of elution procedures could result in good bacteriophage recoveries. The positively charged filters were easy to use, allowed rapid filtration and required minimal manipulation of the sample. The negatively charged filters also gave good adsorption. The best bacteriophage recoveries were achieved when the sample was adjusted to pH 3.5 before filtration. In addition to pH adjustment of the water sample to pH 3.5 before filtration, $MgCl_2$ addition is required. The time required for filtration of 400 ml of natural water samples (30-60 min.) adds significantly to the total time required before data analysis can be accomplished. Further investigation and optimization of the extreme pH ranges involved in this method may enhance the efficiency of recovery and decrease inactivation of bacteriophages. The Bio-Rad 0.2 μm and the Cox 0.46 μm filters gave recoveries indicating potential for bacteriophage concentration by the negatively charged filter technique.

TABLE 7. CONCENTRATION OF BACTERIOPHAGES BY TWO-PHASE POLYMER SEPARATION

Date	Sample Size (mls)	Phase Composition	Ø /100 ml[1] ARCAT	% of Total Recovery[2]					Total Ø Recovered	% Ø Recovered[3]
				Phase 1	Phase 2	Phase 3	Phase 4	Phase 5		
4/4	100	10% Carbowax 1% Dextran SO_4 0.1 M NaCl	86	12.8	23.0	19.4	19.4	23.5	192	228
4/7	100	10% Carbowax 1% Dextran SO_4 0.1 M KCl	48	14.5	16.1	18.5	29.0	21.8	124	258
	100	10% Carbowax 1% Dextran SO_4 0.05 M KCl	48	13.2	11.5	15.5	27.0	32.8	179	363
4/8	100	10% Carbowax 1% Dextran SO_4 0.01 M KCl	19	22.1	19.1	18.6	23.5	16.7	204	1074
	100	10% Carbowax 2% Dextran SO_4 0.01 M KCl	19	15.7	10.8	12.7	28.4	32.4	102	536.8
	50	10% Carbowax 5% Dextran SO_4 0.01 M KCl	19	15.4	30.1	0	15.4	38.5	13	137
4/10	100	10% Carbowax 2% Dextran SO_4 0.05 M KCl	17	23	18	13	27	19	159	935
	100	10% Carbowax 2% Dextran SO_4	17	23	15	13	21	26	136	800
4/17	100	10% Carbowax 2% Dextran SO_4 0.1 M KCl	19	35.3	35.3	17.6	11.8		94.7	498.4

All water samples were taken from the Occoquan Reservoir.
1. Ø - Bacteriophage; 100 ml assayed by ARCAT.
2. Based on number of Ø in the phase compared to the total number of Ø recovered.
3. Based on comparison of ø recovered and Ø in original sample.

TABLE 8. CONCENTRATION OF BACTERIOPHAGES BY MAGNETIC FILTRATION

Date	Water Sample	Ø/100 ml[1] ARCAT	Volume Filtered (ml)	Ø in Filtrate[2]	Ø Recovered	% Ø Recovered[3]
4/14	Raw	2	100	-	0	0
	Raw	2	100	0	0	0
4/15	Raw	3	100	0	0	0
	Raw	3	100	1	0	0
4/16	Raw + $CaCl_2$ 161 ppm	4	200	1	0	0
		4	200	5	0	0
4/21	Raw + $CaCl_2$ 161 ppm	4	200	1	0	0
	Raw + $Al_2(SO_4)_3$ 161 ppm	4	200	0	3	37.5

1. Ø - bacteriophage; 100 ml assayed by ARCAT.

2. pfu/40 ml plated

3. Based on comparison of Ø recovered and Ø in original sample.

- Not evaluated for presence of bacteriophage.

TABLE 9. COMPARISON OF CONCENTRATION METHODS

Concentration Technique	Additional[1] Time Required	% ϕ Recovered	Complexity of Technique[2]
Positive Filters			
50S	30 min	13-73	2
70C	40 min	27-65	2
1MDS	15 min	42-108	2
Negative Filters			
Cox 2 μm	15 min	3-47	3
Cox 0.45 μm	30-60 min	0-125	3
Bio-Rad 0.2 μm	> 2 hr	0-71	3
Osmotic Filtration	8 hr**	0-150	2
Lytic Burst	1 hr	*	2
Adsorption on Inorganic Salts	2 hr	0-9	3
Polymer Two-Phase Separation	18-24 hr	100	4
Magnetic Filtration	1 hr	0-37.5	4

1. Time in addition to that required for ARCAT. (Includes the set-up and performance of the concentration techniques.)

2. Compared to ARCAT at complexity of 1. Methods rated by additional manipulation of samples and materials preparation.

* This technique involves amplification of bacteriophages present, not recovery by concentration.

** At least 8 hours were required to achieve a 2 fold concentration of 100 ml.

Osmotic filtration (dialysis) as a method for concentration of bacteriophages requires little additional sample manipulation and is very simple to execute. However, bacteriophage recoveries were not consistent, ranging from 0 to 400%. The requirement for $\geq$8 hours concentration time prior to analysis by ARCAT is also a disadvantage.

Amplification of bacteriophages by the lytic burst technique is easy to perform, requires no additional equipment and adds only one hour to the test. The disadvantage of this technique is that actual bacteriophage numbers in the original water sample cannot be precisely determined. Amplification is qualitative and determines only the presence or absence of bacteriophages in the original sample.

Adsorption of bacteriophages on inorganic salts did not result in good bacteriophage recovery. The maximum recovery achieved was 5.5% with calcium salts and 9% with aluminum hydroxide. The requirements for washing of the prefilter, preparation of salts solutions, filtration (45-90 min) and centrifugation prior to assay by ARCAT substantially increase the total time required before data are available.

Polymer two-phase separation yielded bacteriophage recoveries generally much greater than 100%. These recoveries probably resulted from lytic bursts induced by the addition of the chemicals for the two-phase separation. Each of the phases in the separation procedure was sampled and no one phase achieved concentration of bacteriophages. This method involves additional time for materials preparation as well as an incubation at 9°C for 18-24 hours. Thus polymer two-phase separation as a means of concentrating bacteriophages did not compare favorably with the other methods evaluated.

When magnetic filtration was employed as a method for concentrating bacteriophages, no bacteriophages were recovered from the magnetite except when 161 ppm of aluminum sulfate were added to the raw water prior to filtration. With the addition of aluminum sulfate, only 37.5% of the bacteriophages present were recovered. The time required to perform magnetic filtration is not a major disadvantage; however, this method was not selected for optimization because of requirements for specialized equipment and poor bacteriophage recoveries.

Three methods were selected for optimization studies based on the criteria listed above. Two of the methods selected were filtration methods utilizing the positively charged Zeta Plus filters and the Bio-Rad and Cox negatively charged filters. The amplification procedure (lytic burst) was the third method selected for optimization. Amplification of coliphages present is accomplished by addition of nutrients and coliform host cells to the sample, incubation to allow bacteriophage replication, and lysis of the host cells prior to assay by ARCAT. This procedure can result in the amplification of one bacteriophage by a factor of 16 to 2200 fold in 60 minutes. These three methods were extensively studied for optimization of conditions to produce consistently high bacteriophage recoveries or amplification of low levels of bacteriophages present in natural waters.

OPTIMIZATION STUDIES

Positive Filters

Based on data obtained in preliminary studies, the 50S Zeta Plus filter gave the best bacteriophage recoveries. Therefore, this filter was used to screen for optimum conditions for adsorption and elution of bacteriophages from positively charged filters. Optimum conditions determined in these studies were evaluated with Zeta Plus filters 60S, 70C, 2X1MDS (double filter) and 3X1MDS (triple filter).

Adsorption--

The optimum pH for adsorption of bacteriophage onto Zeta Plus Filters was determined by seeding raw Occoquan Reservoir water with stock bacteriophage (ATCC #11303, T2 bacteriophage). This bacteriophage was selected at random from stock bacteriophages available at ARC. Seeded samples replaced natural water samples due to an areawide drought which resulted in very low bacteriophage levels in natural water samples. The pH values of the seeded waters were determined and adjusted to the desired pH prior to filtration through Zeta Plus 50S filters. Adsorption of bacteriophages was evaluated by detection of plaque forming units (pfu) in the filtrate by the regular ARCAT method. As shown in Table 10, raw Occoquan samples (natural pH ranging from pH 7.55 to pH 7.8) demonstrated lower adsorption of bacteriophages (as many as 15 pfu observed in 40 ml of filtrate) than Occoquan samples that were pH adjusted before filtration. Adjustment of sample pH to 5.0 or 6.0 before filtration resulted in increased adsorption of the bacteriophages. At pH 6.0, adsorption was approximately 100%; no pfu were observed in filtrates from eleven of fifteen samples filtered at pH 6.0. Adsorption at pH 5.0 was less effective, i.e. 1-3 pfu were detected in 40 ml of filtrate. The time required to filter 500 ml of water sample through a Zeta Plus 50S filter ranged from 15-30 minutes.

Bacteriophage adsorption at pH 6.0 onto 50S Zeta Plus filters was evaluated with respect to a variety of types of seeded water samples and with respect to several stock bacteriophages. Tap water, distilled water, and raw and settled Occoquan waters were sterilized, seeded with a T-even bacteriophage (T2 at an average of 50 pfu/100 ml), adjusted to pH 6.0 and 500 ml volumes were filtered through 50S filters. The results, presented in Table 11, show that bacteriophage T2 in all types of water effectively adhered to the 50S filter. Bacteriophage stocks of T3 (coliphage with short non-contractile tail), ΦX174 (coliphage with no tail) and T2 (coliphage with contractile tail) were titered and diluted in sterile raw Occoquan water to approximately 50 pfu/100 ml when assayed by the regular ARCAT method. These bacteriophages were selected for the study because they represent different coliphage morphological types and the effects of morphology on retention by the filter could be evaluated. Each sample was filtered at pH 6.0. The three bacteriophage types were adsorbed with nearly 100% efficiency, as shown in Table 12. Thus adjustment of water samples to a pH of 6.0 was found to give good bacteriophage adsorption efficiency with the Zeta Plus 50S filter using different water types and different bacteriophage types.

TABLE 10. THE EFFECT OF pH ON ADSORPTION OF BACTERIOPHAGES ONTO 50S FILTERS

Date	pH	Ø Applied/100 ml	Ø/100 ml[1] Filtrate	% Adsorption[2]
5/30	7.73	66	7.5	88.6
	6.0		-	100.0
	7.73		25.0	62.2
	7.73		7.5	88.6
	6.0		2.5	96.2
	6.0		-	100.0
7/1	7.8	49	15.0	69.4
	7.8		37.5	23.5
	7.8		17.5	64.3
	6.0		-	100.0
	6.0		-	100.0
	6.0		-	100.0
	5.0		-	100.0
	5.0		-	100.0
	5.0		-	100.0
7/7	7.75	43	35.0	18.6
	7.75		17.5	59.3
	7.75		32.5	24.4
	6.0		-	100.0
	6.0		-	100.0
	6.0		-	100.0
	5.0		2.5	94.2
	5.0		7.5	82.6
	5.0		2.5	94.2
7/8	6.02	44	2.5	94.3
	6.02		-	100.0
	6.02		5.0	88.6
	6.02		2.5	94.3
	5.0		-	100.0
	5.0		2.5	94.3
	5.0		-	100.0
	5.0		2.5	94.3
7/10	6.0	42	-	100.0
	6.0		-	100.0
	5.0		2.5	94.0
	5.0		5.0	88.0
	6.0		-	100.0
	6.0		-	100.0
	6.0		2.5	94.0

1. Calculated value.
2. % adsorption is based on the total number of pfu in the sample (pfu in 100 ml regular ARCAT).

All samples were 500 ml of sterile raw Occoquan water seeded with T2 bacteriophage

- No coliphage detected in 40 ml of filtrate.

TABLE 11. EFFICIENCY OF ADSORPTION AT pH 6.0 FROM VARIOUS WATERS ONTO ZETA PLUS 50S FILTERS

Date	Sample[1]	Ø Applied/100 ml[2]	Ø/100 ml[3] Filtrate	% Adsorption[4]
7/16	raw	40	-	100.0
	settled	40	2.5	93.8
	tap	40	7.5	81.2
	distilled	40	-	100.0
7/17	raw	28	7.5	73.0
	settled	37	-	100.0
	tap	25	7.5	70.0
	distilled	15	-	100.0

1. All samples were 500 ml of sterile Occoquan water seeded with ATCC stock bacteriophage T2.
2. Ø - bacteriophage.
3. Calculated value.
4. Percent adsorption is based on the number of Ø applied as determined by ARCAT.

\- No coliphage detected in 40 ml of filtrate.

TABLE 12. EFFICIENCY OF BACTERIOPHAGE ADSORPTION AT pH 6.0

Date	Ø[1,2]	Filter	Ø Applied/100 ml	Ø/100 ml[3] Filtrate	% Adsorption[4]
7/23	T2	50S	26	-	100.0
	T2	60S	26	-	100.0
	ΦX174	50S	9	2.5	72.2
	ΦX174	60S	9	-	100.0
	T3	50S	13	-	100.0
	T3	60S	13	-	100.0

1. Ø - bacteriophage.
2. All Ø types were diluted in 500 ml sterile raw Occoquan water.
3. Calculated value.
4. Percent adsorption is based on the number of Ø applied as determined by ARCAT.

\- No coliphage detected in 40 ml of filtrate.

Elution--

Glycine, trypticase soy broth (TSB) and beef extract were evaluated as eluents for removal of bacteriophages adsorbed onto Zeta Plus 50S filters. Eluent pH values and concentrations were varied. Glycine was evaluated at molarities of 0.05, 0.01, 0.005 and 0.001 and at pH values of 9.0 and 11.0. Recovery of adsorbed coliphages never exceeded 60% as shown in Table 13.

Beef extract was evaluated as an eluent alone and with addition of antifoam agent, Dow Corning Anti-Foam Emulsion B. Seeded sterile raw waters at pH 5.0-7.4 were filtered through 50S filters and elutions were performed with 3% beef extract at pH 8.0 and 9.0 in the presence and absence of antifoam (50 mg/L). Beef extract without antifoam demonstrated a recovery of 59-87% of adsorbed bacteriophages. As shown in Table 14, the recoveries with beef extract plus antifoam ranged from 83 to 107%. The presence of the antifoam agent increased the percentage of the bacteriophage recovered over that of the beef extract alone. The lower bacteriophage recovery with the beef extract eluent containing no antifoam agent was probably due to trapping of the bacteriophage in the foam bubble. However, even with the antifoam agent added, the beef extract tended to form foam. The presence of the foam bubbles and the darker color of the agar (due to the presence of the beef extract) made enumeration of the coliphages difficult. Thus, in spite of the good bacteriophage recoveries obtained with beef extract with antifoam, it is not the ideal eluent for use with the ARCAT test.

TSB was evaluated as an eluent with the 50S filter at pH values of 8.0, 8.5, 9.0 and 11.0. As shown in Table 15, recovery of adsorbed bacteriophages was 70 to 90% for pH values ranging from 8.0 to 9.0. At pH 11.0, bacteriophage recovery dropped to 40%. The use of TSB eliminated the problems of dark plates and bubbles found with the beef extract eluate while maintaining good recovery. Thus, TSB at pH 8-9 is the preferred eluent for removal of bacteriophages from the Zeta Plus filters.

Comparison of Zeta Plus Filters--

Once bacteriophage filtration and recovery techniques were optimized, a variety of Zeta Plus filters were evaluated to determine if increased filtration/recovery efficiency could be achieved. The filters evaluated were Zeta Plus 50S, 60S, 70C and 1MDS. The 1MDS was evaluated in double and triple layers. For this study, sterile raw Occoquan water was seeded with bacteriophage ΦX174, T3 or T2. In all cases, a 500 ml volume of the seeded water, adjusted to pH 6.0, was passed through each filter. The filters were eluted with TSB at pH 8.4 or 9.0.

The results of the filter comparison tests are presented in Table 16. The double layer 1MDS had low adsorption and recovery with bacteriophage T2. Triple layers of 1MDS filter increased the number of bacteriophages adsorbed and pH 9 gave increased recovery. Based on low recoveries achieved with the 1MDS filters and the increased cost of using double or triple filter layers, the 1MDS filter was not further evaluated. The 50S filter gave between 93 and 100% adsorption of bacteriophage T2 and recoveries ranged from 72 and 88%. However, for bacteriophage ΦX174, adsorption onto 50S filters was low, 31.8%, and recovery was only 63%. Adsorption of T3 bacteriophages onto the 50S filter was 100%, but recovery

TABLE 13. GLYCINE AS AN ELUENT FOR ADSORBED BACTERIOPHAGES

Date	Ø[1]	Eluent Molarity	Eluent pH	Ø Applied[2]	Ø Adsorbed[3]	Ø in Eluate[4]	% Recovery[5]
6/30	ARC 1	0.05	11.0	240	240	93	38.8
		0.01	11.0	240	240	115	47.9
		0.005	11.0	240	240	115	47.9
7/8	T2	0.01	11.0	220	208	118	56.7
		0.01	9.0	220	220	113	51.4
		0.005	11.0	220	196	75	38.3
		0.01	11.0	220	220	91	41.4
		0.01	9.0	220	208	84	40.4
		0.05	11.0	220	220	72	32.7
		0.05	9.0	220	208	43	20.7

Ø - bacteriophage

1 Both Ø types were diluted in 500 ml of sterile raw Occoquan water and filtered through Zeta Plus 50S filters.

2 Determined by ARCAT.

3 Determined by total Ø applied and Ø detected in filtrates.

4 Eluate volumes were monitored and entire volume was used to determine Ø in the eluate.

5 Percent recovery is based on total Ø adsorbed and Ø recovered in eluate.

TABLE 14. BACTERIOPHAGE ELUTION FROM THE ZETA PLUS 50S FILTER WITH BEEF EXTRACT

Date	Sample pH[1]	Ø[2]/100 ml ARCAT	Ø[3] Adsorbed	Eluent[4]	Eluent pH	Ø in Eluate	% Recovery[5]
7/9	6.0	42	210	3% BE	9.0	123	58.6
	6.0	42	210	3% BE	8.0	150	71.4
	5.0	42	198	3% BE	9.0	147	74.2
	5.0	42	185	3% BE	8.0	160	86.5
7/28	6.0	43	215	3% BE 50 mg/L AF	8.0	179	83.3
7/30	6.0	45	225	3% BE 50 mg/L AF	8.0	241	107.1
	6.0	45	213	3% BE 50 mg/L AF	9.0	191	89.7

1 500 ml samples at the specified pHs were seeded with coliphage T2 in raw sterilized water from the Occoquan Reservoir and filtered.
2 Ø - bacteriophage, T2; 100 ml assayed by ARCAT.
3 Ø adsorbed is based on Ø detected in filtrate.
4 Eluents: BE= beef extract, AF = antifoam agent (Dow Corning for Fisher CS283-4 - Anti-foam B emulsion).
5 Percent recovery is based on total Ø adsorbed and Ø recovered in the total volume of eluate collected (~ 20 ml).

TABLE 15. BACTERIOPHAGE ELUTION FROM ZETA PLUS 50S FILTER WITH TRYPTICASE SOY BROTH (TSB)

Date	Sample pH	Ø[1]/100 ml ARCAT	ml Filtered	Ø Adsorbed	Eluent pH	Ø in Eluate	% Recovery[2]
7/7	6.0	43	500	215	8.0	174	80.9
	6.0	43	500	215	9.0	154	71.6
	6.0	43	500	215	11.0	86	40.0
	5.0	43	500	203	8.0	138	68.0
	5.0	43	500	178	9.0	118	66.3
	5.0	43	500	203	11.0	77	37.9
7/10	6.0	42	500	210	8.0	168	80.0
	6.0	42	500	210	8.5	162	77.1
	6.0	42	500	198	9.0	175	88.4
7/14	6.0	47	500	223	8.0	161	72.2

Bacteriophage T2 in sterile raw Occoquan water was used for all experiments.

1 Ø - bacteriophage; 100 ml assayed by ARCAT.

2 Percent recovery based on total Ø adsorbed to the filter.

TABLE 16. COMPARISON OF ZETA PLUS FILTERS FOR ADSORPTION AND ELUTION OF BACTERIOPHAGES

Date	ø[1]	Sample pH	ø/100 ml[2] ARCAT	Filter	ml Filtered	ø[3] Adsorbed	% Adsorbed	Eluent	ø in Eluate	% Recovery[4]
7/14	T2	6.0	47	50S	500	223	94.9	TSB (pH8)	161	72.2
			47	2X1MDS	500	35	14.9	TSB (pH8)	12	34.3
			47	70C	500	235	100.0	TSB (pH8)	119	50.6
7/16	T2	6.0	40	50S	500	200	100.0	TSB (pH9)	177	88.5
			40	60S	500	200	100.0	TSB (pH9)	200	100.0
			40	70C	500	200	100.0	TSB (pH9)	122	61.0
			40	3X1MDS	500	175	87.5	TSB (pH9)	133	76.0
7/30	T2	6.0	76	50S	500	355	93.4	TSB (pH8.4)	260	73.2
			76	60S	500	355	93.4	TSB (pH8.4)	330	92.9
	ΦX174	6.0	11	50S	500	18	32.7	TSB (pH8.4)	11	61.1
			11	60S	500	43	78.2	TSB (pH8.4)	43	100.0
	T3	6.0	23	50S	500	115	100.0	TSB (pH8.4)	7	6.1
			23	60S	500	103	89.6	TSB (pH8.4)	13	12.6

1 ø - bacteriophage.

2 100 ml assayed by ARCAT.

3 ø adsorbed is based on total ø applied to the filter.

4 Percent recovery is based on total ø adsorbed to the filter and ø recovered in the eluate.

One sample of each ø, filter and eluent pH was analyzed in these comparisons of positively charged filters.

was extremely poor, only 6.1%. The 70C filter gave good adsorption of bacteriophage T2. Recovery, however, was only 60% and this filter was not further evaluated. Adsorption and recovery of bacteriophage T2 from the 60S filter was between 90 and 100%. With ΦX174, adsorption was 77% and recovery was 100% which indicates an acceptable concentration method for this bacteriophage. With bacteriophage T3, adsorption was 90%, however, recovery was only 13%. These results indicate that conditions for T3 (and perhaps other T-odd coliphages) elution from the filter have not been optimized.

Overall the 60S filter gave the best coliphage adsorption/recovery. The 50S filter also gave good adsorption/recovery. None of the filters tested gave good recovery of T3 bacteriophage under the elution conditions described.

Negative Filters

The Cox 0.45 µm and the Bio-Rad 0.2 µm negatively charged filters were selected for intensified study based on the results of the preliminary experiments. However, Bio-Rad discontinued manufacture of the 0.2 µm filters, so optimization experiments were performed using only the Cox 0.45 µm filter. Optimum pH for adsorption was determined and eluents were evaluated.

Adsorption--

Stock bacteriophage (T2) was added to sterile raw Occoquan Reservoir water at approximately 50 pfu/100 ml. Five hundred ml volumes of the seeded samples at natural pH and at pH values adjusted to 3.5, 4.5 and 5.0 were filtered through the Cox 0.45 µm filter to determine the optimum pH for adsorption. As indicated in Table 17, adsorption of 90 to 100% occurred at pH 5.0 in 11 of 12 samples monitored. Time required for filtration of 500 ml of water sample ranged from 90 to 140 minutes.

Elution--

Glycine and trypticase soy broth (TSB) were evaluated for elution of bacteriophage T2 from the Cox 0.45 µm filters. In the preliminary studies, the presence of 0.1 M glycine inhibited the growth of the E. coli C bacterial lawn. Therefore, the concentration of the glycine in the eluent was maintained at 0.01 M for the optimization studies. The bacteriophage recoveries using the 0.01 M glycine or TSB adjusted to pH 8.0, 9.0 or 11.0 are presented in Table 18. Trypticase soy broth at pH 9.0 and glycine at pH 9.0 and 11.0 gave similar recoveries. However, in contrast to the relatively narrow range of recovered bacteriophage using TSB as an eluent for the Zeta Plus filters (70% to 90% at elution pH 8-9, adsorption at pH 6), recovery of bacteriophages from negatively charged filters was highly variable with both eluents. Recoveries from the filters ranged from 35% to 148% with TSB elution at pH 8.0 and pH 9.0 after adsorption at pH 5.0, and 68% to 133% with glycine elution at pH 9.0 and pH 11.0 after adsorption at pH 5.0.

Bacteriophage recoveries of $\leq$10% on samples that had 100% adsorption (samples adjusted to pH 3.5 and 4.0) suggested inactivation of the bacteriophages. A brief study of bacteriophage activity after exposure to pH 3.5 in an aqueous environment for 90 minutes was conducted to confirm this observation.

TABLE 17. THE EFFECT OF pH ON ADSORPTION OF BACTERIOPHAGES ONTO THE COX 0.45 µm NEGATIVE FILTER

Date	pH	Ø[1]/100 ml Applied	pfu/40 ml Filtrate	Ø/100 ml[2] Filtrate	% Adsorption[3]
8/1	3.5	44	0	-	100
	4.0	44	1	2.5	94.3
	5.0	44	0	-	100
8/5	8.6	26	10	25.0	3.9
	5.0	26	0	-	100
	5.0	26	0	-	100
	5.0	26	1	2.5	90.4
	5.0	26	0	-	100
	5.0	26	0	-	100
	5.0	26	1	2.5	90.4
	5.0	26	0	-	100
8/6	5.0	82	0	-	100
	5.0	82	2	5.0	93.9
8/13	5.0	52	10	25.0	51.9

The sample contained the T2 coliphage.

1. Ø - bacteriophage; 100 ml assayed by ARCAT.
2. Calculated value.
3. Based on total bacteriophages applied to the filter in 500 ml.

TABLE 18. ELUTION OF BACTERIOPHAGES FROM THE COX 0.45 μm NEGATIVE FILTER

Date	Sample[1] pH	Eluent[2]	Eluent pH	Ø[3] Applied	Ø Adsorbed	Ø in Eluate	% Recovered[4]
8/1	3.5	TSB	9.0	220	220	19	8.6
	4.0	TSB	9.0	220	208	22	10.6
	5.0	TSB	9.0	220	220	78	35.5
8/5	8.6	TSB	9.0	130	10	0	0
	5.0	TSB	9.0	130	130	152	116.9
	5.0	TSB	11.0	130	130	92	70.8
	5.0	TSB	8.0	130	123	182	148.0
	5.0	Glycine	8.0	130	130	98	75.4
	5.0	Glycine	9.0	130	130	153	117.7
	5.0	Glycine	11.0	130	123	163	132.5
8/6	5.0	TSB	9.0	410	410	323	78.8
	5.0	Glycine	9.0	410	385	262	68.0

1. Sample used was T2 in sterile, raw Occoquan water.
2. TSB, 3%; Glycine, 0.01 M.
3. Ø - bacteriophage.
4. Based on total Ø adsorbed to filter and Ø eluted.

For this experiment, a water sample was divided into two parts. One half of the sample was adjusted to pH 3.5 and allowed to remain at this pH for 90 minutes. The pH of this sample was then restored to its natural pH before plating. The sample exposed to pH 3.5 produced 25 pfu versus 49 pfu in the sample at natural pH. Thus, the low pH appeared to inhibit plaque formation and resulted in lower bacteriophage recovery.

Comparison of Zeta Plus and Cox Filters for Concentration of Bacteriophages

In the optimization studies, the positively and negatively charged filters were used to concentrate bacteriophages from a variety of water samples. Comparison of the data obtained indicates that the Zeta Plus 60S filters are better suited for concentration of bacteriophages for the ARCAT test than the Cox 0.45 µm filters. There are several reasons for this choice.

- The optimum pH for adsorption on the Zeta Plus 60S filter is 6.0 compared to 5.0 for the Cox 0.45 µm filter. Thus, the positive filters require less radical sample pH adjustment and a lower potential for bacteriophage inactivation during the adsorption step.

- Good elution recoveries were obtained from both filters using TSB at pH 8.0 to 9.0. However, the recoveries from the Zeta Plus filters were more consistent than those observed from the Cox 0.45 µm filter.

- The filtration time for the Zeta Plus 60S filter was 30 minutes versus 90 minutes required for filtration with the Cox 0.45 µm filter.

Based on these data, the Zeta Plus 60S filter was selected for the verification studies.

Lytic Bursts

Optimization and control of the lytic burst resulting from the infection of one E. coli host cell by one bacteriophage could result in a system which would permit detection of low levels of bacteriophage (i.e. 1 pfu/100 ml) in natural water samples. Bacteriophages present in a natural water sample may be amplified by replication as a result of the addition of nutrients and the E. coli C host to the water sample. Experiments were conducted to examine the possibility of estimating the number of bacteriophages originally present in the water sample by extrapolation from data on bacteriophage burst size and lytic cycle time during the amplification procedure. Data were obtained on burst sizes and lytic cycle times for two coliphages, ΦX174 and T3.

One-Step Growth Curve--

ΦX174 and T3 bacteriophages represent two different coliphage morphological types found in natural water samples. These bacteriophages were studied extensively to determine the average burst size for each bacteriophage and the average lytic cycle time using the one-step growth experiment.

The results obtained with the one-step growth experiment to determine burst time and burst size for bacteriophage ΦX174 are presented in Table 19 and

Figure 6. In this experiment, the time required for completion of the lytic cycle (time when the pfu/ml plateau is reached) was 35 minutes. The number of adsorbed bacteriophages was calculated from the pfu/ml present at 18 and 25 minutes. The average pfu at the plateau (2.5×10^{10}) divided by the number of bacteriophage (pfu/ml) initially adsorbed (8.8×10^{8}) gave a burst size of 28. If samples had been taken from the second growth tube at 27, 30 and 32 minutes, increasing numbers of pfu/ml would have demonstrated a "rise" period, since not all infected cells burst at the same time. If samples had been taken beyond 60 minutes, a second rise and burst could have been demonstrated.

Five one-step growth experiments were performed with ΦX174 resulting in an average burst size of 24. The average burst time ranged between 45 and 60 minutes. A summary of the data obtained in these experiments is presented in Table 20.

One-step growth experiments performed with T3 indicated coliphage burst sizes that ranged from 57-100 and burst times consistently between 30 and 40 minutes. Results of these experiments are presented in Table 21.

Single Burst--

In a natural water sample, the number of bacteriophages present can be very low. An experiment that is analogous to a natural water system (i.e. very low pfu/ml) is the single burst experiment. This experiment was used to determine the average burst size of bacteriophages ΦX174 or T3 knowing the burst time from the one-step growth experiment. The results of the single burst experiment with bacteriophage T3 are presented in Table 22.

In a single burst experiment, when each tube contains on the average less than one infected bacterium, the Poisson distribution (a statistical method for determining number of events per sample when the average number of events is very small) can be used to determine the burst size. Applying the Poisson distribution to the data in Table 22, a burst size of 79 was determined for T3. Five single burst experiments were performed with T3 and a summary of the data obtained in these experiments is presented in Table 23.

Six single burst experiments were also performed with ΦX174. The range of burst sizes was 18-37, with 67% of the burst sizes falling between 22 and 29. The results of these ΦX174 single burst experiments are presented in Table 24.

Burst size is the mean number of phage particles liberated per infected bacterium. The range of variation in plaque counts from the single burst tubes indicated the range of burst sizes to be expected from individual infected cells. The burst size is influenced by the length of the latent period; generally the longer the time before the lytic burst, the larger the number of bacteriophages released by the infected cell. The average burst size was 93.4 pfu and 27.2 pfu for T3 and ΦX174, respectively. The range of burst sizes calculated varied by approximately 35%.

Laboratory Amplification--

The addition of nutrients and a suitable titer of E. coli C host cells to

TABLE 19. ΦX174 ONE STEP GROWTH EXPERIMENT*

Time (Minutes)	First Growth Tube (Average pfu of duplicate samples)	Second Growth Tube (Average pfu of duplicate samples)
18	44	0.0
25	44	1.0
35	too numerous to count	13.0
40	too numerous to count	13.0
45		13.5
50		17.0
55		14.5
60		16.5

* m.o.i. = 0.1

TABLE 20. SUMMARY OF ΦX174 ONE STEP GROWTH EXPERIMENT DATA

Date	Multiplicity of Infection	Burst Time (minutes)	Burst Size (pfu)
7/28	0.08	60-65	28
7/29	0.06	50-60	24
8/14	0.08	40-50	22
8/20	0.01	35-48	14
8/26	0.1	35-60	34

TABLE 21. SUMMARY OF T3 ONE STEP GROWTH EXPERIMENT DATA

Date	Multiplicity of Infection	Burst Time (minutes)	Burst Size (pfu)
8/11	0.08	30-45	100
8/12	0.06	35-40	98
8/28	0.08	35-40	57

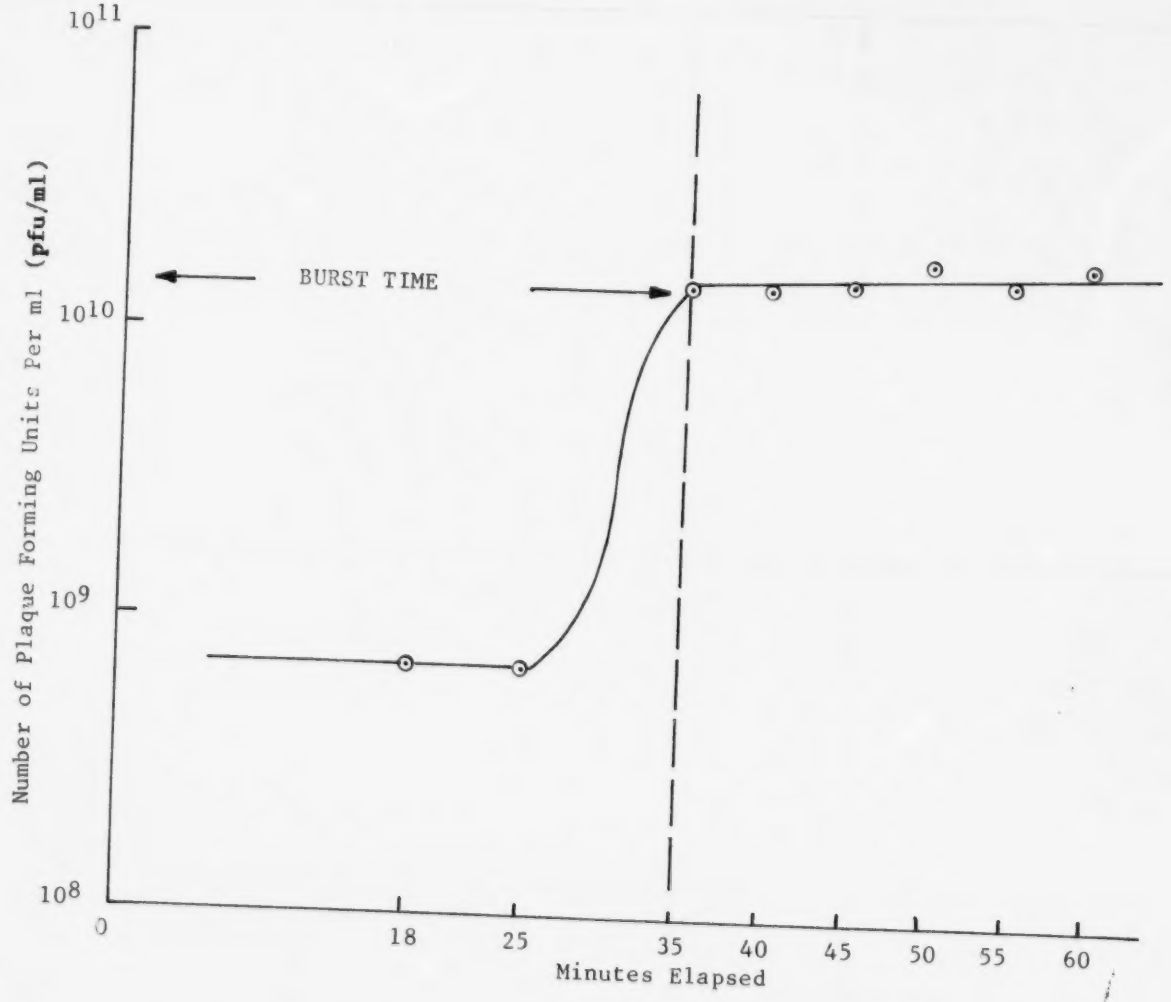


Figure 6. One-Step Growth Curve for ΦX174

TABLE 22. RESULTS OF T3 SINGLE BURST EXPERIMENT

Tube	pfu
1	0
2	71
3	0
4	0
5	64
6	0
7	0
8	0
9	84
10	142
11	133
12	85

TABLE 23. SUMMARY OF T3 SINGLE BURST DATA

Date	Multiplicity of Infection	Burst Size (pfu)
8/14	0.07	84
8/14	0.07	59
8/15	0.07	116
8/17	0.07	129
8/19	0.1	79

TABLE 24. SUMMARY OF ΦX174 SINGLE BURST DATA

Date	Multiplicity of Infection	Burst Size (pfu)
8/1	0.01	29
8/2	0.01	29
8/4	0.01	18
8/6	0.08	22
8/7	0.08	28
8/8	0.09	37

a water sample enhances adsorption of bacteriophages present in the sample and can result in replication of the bacteriophage in the infected cell. Incubation of the mixture at 35°C for a set time, based on the one-step growth burst time data, will amplify the bacteriophage(s) present. After amplification, samples can be plated, incubated and the pfu counted. Theoretically the original number of pfu in the water sample can be estimated from the amplified count, based on burst sizes of ΦX174 and T3 as determined in earlier experiments. Before attempting to amplify a natural water sample, amplification of low titers of ΦX174 and T3 were performed to compare the burst time and burst size in the amplification method with burst time and burst size determined by one-step growth and single burst experiments.

In the one-step growth and single burst experiments, the E. coli C host was added to the bacteriophage samples and dilutions were made from this adsorption mixture. In these experiments, the m.o.i. ranged from 0.01-0.1. However, in the laboratory amplification, the bacteriophages were present at very low levels when the E. coli C was added, thus the m.o.i. was very low (i.e. 3 pfu/ml with 1 x 10^7 E. coli/ml equals a m.o.i. of 3 x 10^{-7}). This laboratory amplification was analogous to mixing a natural water sample containing 1 pfu/100 ml of water with 1 x 10^7 cfu E. coli C/ml (m.o.i. of 1 x 10^{-9}). In the laboratory amplification experiments with ΦX174 and T3, the m.o.i. ranged from 3 x 10^{-8} to 6 x 10^{-8}. As shown in Table 25, the burst times for ΦX174 during the amplification studies fell within the range of the burst times determined in the one-step growth experiments (~ 45 min). Although the burst size showed more variation than the previous experiments (low of 5 and a high of 30), three of five values were within a 4 pfu range (26, 27 and 30 pfu).

In amplification studies with T3 (Table 25), the burst times were the same as found earlier, approximately 40-45 minutes. The burst sizes ranged from 60-113, with an average burst size of 89.

Laboratory amplification of ΦX174 and T3 demonstrated little change in burst size or burst time when the m.o.i. was very low. The amplification results are compared with one-step growth and single burst experiments in Table 26.

Laboratory amplification of ΦX174 and T3 took place in the absence of other bacteriophage types. Natural water samples are likely to contain more than one bacteriophage type and laboratory amplification experiments were conducted using a mixture of ΦX174 and T3 to determine any effect of the mixture on burst time or burst size. In the first mixed bacteriophage experiment, ΦX174 had a burst size of 30 pfu and a burst time between 45 and 50 minutes. A burst size of 88 pfu was determined for T3 with a burst time of 40-45 minutes. The average burst size from the mixture was 133 pfu with a burst time of 45 minutes. Burst time and burst size did not appear to be significantly altered by the presence of two different bacteriophages since addition of the single bacteriophage burst sizes of 30 pfu and 88 pfu results in a total of 118 pfu compared to a total in the mixed flask of 133 pfu. A second amplification experiment with the bacteriophage mixture demonstrated similar results.

The laboratory amplification studies indicated that although burst sizes of the two bacteriophages (T3 and ΦX174) differed by a factor of approximately 3, the burst times were similar. Thus, in amplification of a raw water sample, a burst should occur before 50 minutes assuming the presence of ΦX174 and/or T3.

TABLE 25. SUMMARY OF ΦX174 AND T3 AMPLIFICATION DATA

Date	Bacteriophage	Multiplicity of Infection	Burst Time (Minutes)	Burst Size (pfu)
8/22	T3	$3x10^{-8}$	40-45	113
8/25		$3x10^{-8}$	40-50	104
8/25		$3x10^{-8}$	40-45	88
8/26		$3x10^{-8}$	40-45	71
8/27		$4.8x10^{-8}$	29-35	60
8/28		$4.8x10^{-8}$	33-41	92
8/25	ΦX174	$6x10^{-8}$	55-65	17
8/25		$6x10^{-8}$	45-50	30
8/26		$6x10^{-8}$	45-55	5
9/9		$3.7x10^{-8}$	40-45	26
9/9		$3.7x10^{-8}$	30-35	27

TABLE 26. COMPARISON OF BURST SIZE AND BURST TIME OF ΦX174 AND T3 BY ONE STEP GROWTH, SINGLE BURST AND AMPLIFICATION TECHNIQUES

	One Step Growth	Single Burst	Laboratory Amplification
ΦX174 average burst time (minutes)	44-57	-	43-49
ΦX174 average burst size (pfu)	24	27	21
T3 average burst time (minutes)	33-42	-	37-44
T3 average burst size (pfu)	85	92	89

- No burst time determined in Single Burst Experiments.

Amplification of Natural Water Samples--

The first attempted amplifications using raw water were performed on samples from Accotink Creek (a small creek fed from Lake Accotink). Nutrients were not added to the samples. Samples were plated at 5 minute intervals after 30 to 50 minutes of incubation with the host culture. By the end of the 50 minute period, there were about the same number of pfu on the 50 minute plates as on the 30 minute plates, indicating that a burst had not occurred. Therefore, to determine if a burst did occur, two of the flasks were sampled again after 6 hours of incubation. By 6 hours, a burst had occurred. The very long burst time was believed to be due in part to the lack of added nutrients in the water. Without added TSB (or some other form of nutrients), only one replicative cycle takes place and that cycle occurs very slowly. Once the nutrients stored in the bacterial cells are depleted, replication stops.

A second amplification procedure was performed on a sample of water from Accotink Creek. The procedure was the same as in the earlier amplification, except 3 grams of dry TSB were added to each flask. (The TSB used in earlier experiments as a diluent was also a 3% solution.) Samples were taken up to 60 minutes and plated. Again there was no appearance of a burst. Therefore, at 180 minutes another 4 samples were plated. The 180 minute pfu count indicated a burst of 35 pfu.

These two raw water amplifications produced bursts much later than expected. However, since samples were not taken between 50 minutes and 180 minutes (3 hours), the actual time of the burst could not be determined. In order to pinpoint when the burst was taking place, the next amplification experiment was performed on a 200 ml sample of water with 6 g of TSB and 4 ml of E. coli C added to allow for extensive sampling. Several 100 ml samples were also prepared and sampled once as in the previous experiments. Two water sources were used, Accotink Creek and Cherokee Creek (small creek near Atlantic Research in Alexandria, Virginia).

The pfu remained fairly constant for the Accotink Creek sample up to 80 minutes, ranging from 0-20 pfu/100 ml. At 100 minutes and 120 minutes, there were 220 pfu/100 ml and 600 pfu/100 ml, respectively. Obviously a burst occurred, but the burst size could not be determined since there was no plateau.

Amplification of Cherokee Creek water yielded irregular pfu counts up to 80 minutes, and a plateau of 880 pfu/100 ml at 100 minutes and 870 pfu/100 ml at 120 minutes. Again, a burst occurred, but the size could not be determined.

Based on results from these experiments, amplification of raw water appeared to present two major problems, i.e. the late burst time, and the inconsistent early counts. To determine the cause of the problems, the following variables were examined:

- The effect of dry nutrients compared to concentrated liquid nutrients
- The effect of fresh E. coli C host versus frozen and thawed E. coli C

- The E. coli C titer
- The effect of rain, or lack of it, on the type(s) of bacteriophage present
- Effect of pH on adsorption of bacteriophage

The first variable examined was the effect of dry nutrients compared with liquid nutrients. Liquid, autoclaved TSB was used as a source of nutrients in the one-step growth, single burst and laboratory amplification experiments. Dry TSB was added for nutrients in the raw water experiments. To determine the effect of these nutrients on burst time the following experiments were performed.

Two flasks, each containing 100 ml of water from Cherokee Creek, were prepared. Three grams of dry TSB were added to one flask and a solution of 10X TSB (10 times more concentrated than usual) was prepared and added to the second 100 ml water sample in a ratio of 1:10. Thus, the final concentration of the nutrients (3% TSB) in the water was the same as used in the one-step growth experiments. Two ml of E. coli C (thawed frozen host) were added to each flask, the flasks were incubated at 35°C and two 5 ml samples were taken at intervals from 50 to 130 minutes. The samples were plated, incubated in the usual manner, plaques counted, and the burst times determined.

Data showed that the use of dry TSB or 10X TSB resulted in a burst at 125-130 minutes. Good data were obtained on early counts and in both cases a plateau was reached so that a burst size could be determined. The calculated burst size for each flask was 16 pfu. These data indicate there was no difference in burst size or burst time when dry TSB or 10X TSB was used as the nutrient source.

The second variable examined was the effect of using a fresh E. coli C host culture versus the frozen and thawed E. coli C host. The E. coli C host culture used in experimental work on the ARCAT procedure is prepared in large batches, distributed in vials of 4.5 ml each and frozen. Vials of host E. coli C are then thawed and used as needed. Since environmental conditions affect the burst size and burst time of the bacteriophage, an experiment was performed to compare the burst sizes and times using fresh E. coli C suspensions versus frozen and thawed E. coli C suspensions. In this experiment, a one-step growth experiment was performed with ΦX174 using freshly made E. coli C rather than the usual frozen and thawed E. coli C host. The results showed no difference between burst size and burst time when using frozen and thawed or fresh E. coli C. The burst size using the fresh E. coli C was 25 pfu with burst time between 40 and 60 minutes. An average burst size of 24 pfu and a burst time between 44 and 57 minutes were obtained when frozen host was used. Experimentation indicated that the E. coli C host must be thawed at 44.5°C for 10-15 minutes before use. Thawing for less than 10 minutes resulted in no burst.

The third variable examined was the effect of the E. coli C host titer on raw water amplification burst delays. It is possible that with so few bacteriophage present in a raw water sample, more E. coli C may be needed to enhance adsorption for replication to take place. Rather than use a raw water sample for this experiment, a low titer of stock ΦX174 was used. Two flasks were prepared: E. coli C host culture was added to yield ~1 x 10^7 cfu/ml in flask 1

and ~1 x 10^8 cfu/ml in flask 2. The flask with 1 x 10^7 cfu E. coli C/ml produced a burst of 26 pfu in 40-45 minutes while the 1 x 10^8 cfu E. coli C/ml flask produced a burst of 27 pfu in 30-35 minutes. This experiment showed that although the burst size was not changed by the addition of more E. coli C, the burst occurred 10 minutes earlier. However, in order to add sufficient E. coli C to 100 ml of water, 20 ml of E. coli C (~1 x 10^9 cfu/ml) must be added.

To evaluate the effect of the E. coli C titer in raw water amplification, a series of experiments was performed in which the effects of E. coli C host concentration of~1 x 10^7 cfu/ml and~1 x 10^8 cfu/ml on amplification of bacteriophages in natural waters were determined. The experiments were performed as follows: 200 ml of natural water (Potomac River or Accotink Creek) were pipetted into each of 2 sterile flasks. The standard 3% concentration of nutrients was achieved by the addition of 6 g TSB to each flask. The E. coli C host was thawed at 44.5°C for 15 minutes and the flasks containing the water sample and TSB were prewarmed for 15 minutes at 35°C. The warmed E. coli C stock (4 ml) was added to one flask to yield approximately 2 x 10^7 cfu/ml while 40 ml of E. coli C stock were added to the second flask to achieve approximately 2 x 10^8 cfu/ml. The flasks were incubated at 35 + 0.5°C for 120 minutes. Duplicate 5 ml samples were removed at selected intervals from each flask, plated in ARCAT and scored for pfu after 4-6 hours. Because of the presence of very tiny plaques during periods of little or no rain, the pfu were scored according to size as well as for total pfu/100 ml. The "tiny" plaques were no larger than pinpoints on the bacterial lawn. The "small" plaques were larger than the pinpoint plaques with diameters of up to 1 mm. The "medium" plaques ranged in diameter from 1 mm to 2 mm.

The results of these experiments demonstrated that in all cases the bursts took place earlier when there were 1 x 10^8 cfu E. coli C/ml present. The burst time for the "tiny" pfu occurred at 55-80 minutes with the higher E. coli C titer, whereas the burst time was 85-100 minutes at the lower E. coli C titer. The results suggest that these "tiny" pfu are produced by different bacteriophage than those observed in earlier experiments which required 120 minutes for a burst to occur. The results of a representative experiment are presented in Figures 7 and 8.

A second striking difference in the effect of the E. coli C titers was the difference in morphology of the plaques. In the flasks with E. coli C at approximately 1 x 10^7 cfu/ml, most of the plaques after amplification were of the "tiny" size. Although "small" and "medium" plaques were noted in early counts, they were not in increased numbers following amplification (Figure 7). The flasks containing approximately 1 x 10^8 cfu E. coli C/ml demonstrated amplification of the "small" and "medium" as well as the "tiny" plaques (Figure 8). All bacteriophages produced bursts between 55 and 80 minutes.

These experiments clearly indicate that the higher E. coli C titer (1 x 10^8 cfu/ml) optimized the burst time and produced the conditions necessary for amplification of a variety of bacteriophages which resulted in varying size plaque formation. The use of higher E. coli C host titers in amplification flasks is not feasible. Growth of the E. coli C host culture beyond $O.D._{530}$ of 0.55 does not result in optimal plaque formation. Thus to obtain a denser culture large quantities of host culture would have to be concentrated by centrifugation before addition to the amplification flasks.

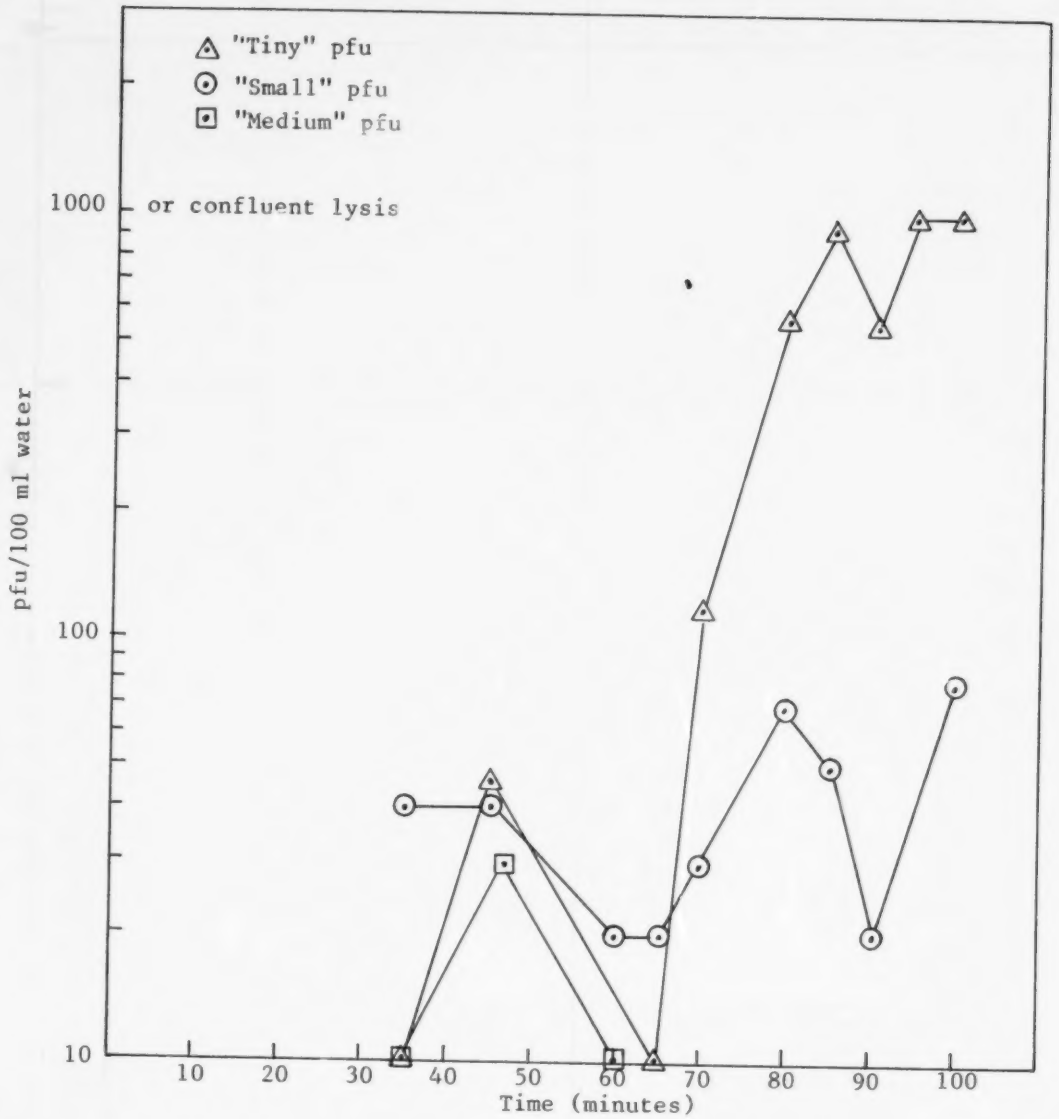


Figure 7. Results of Potomac River Amplification Using $1x10^7$ cfu E. coli C/ml

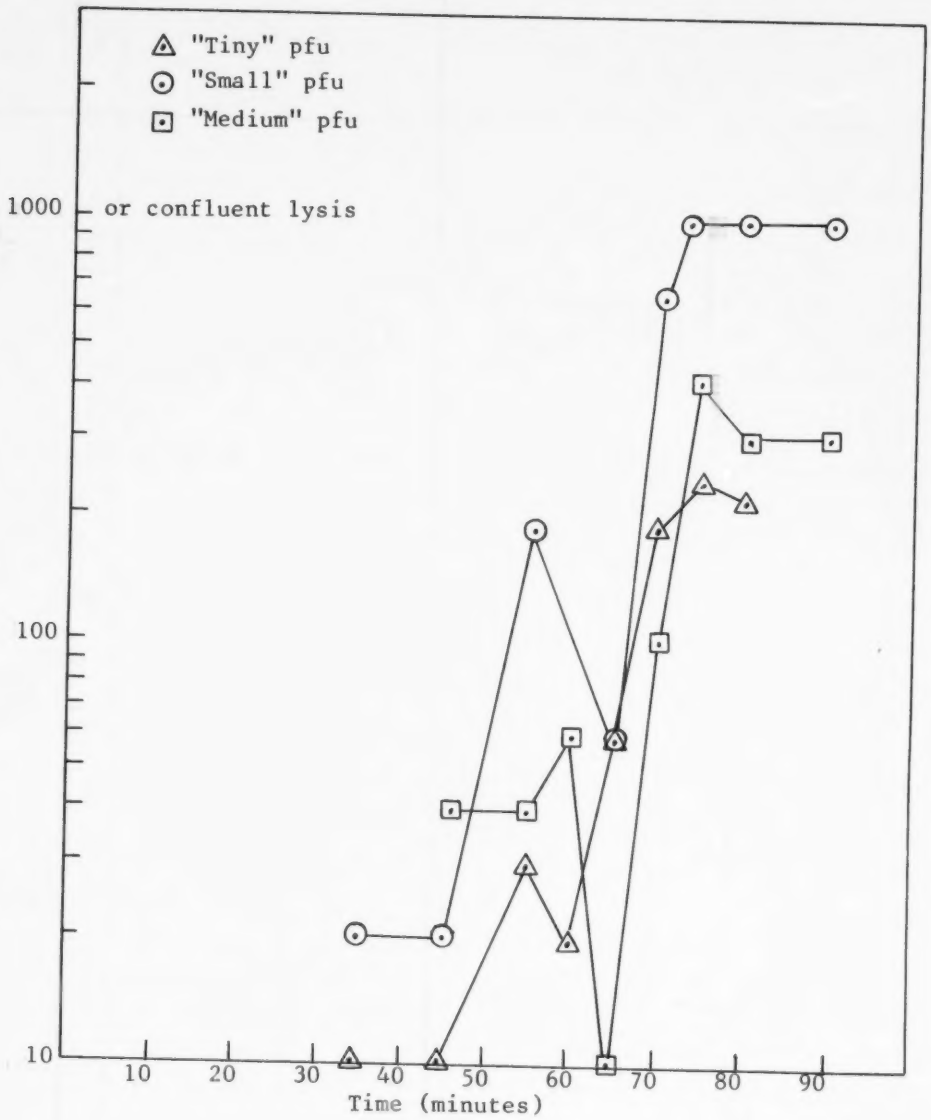


Figure 8. Results of Potomac River Amplification Using $1x10^8$ cfu E. coli C/ml

A fourth possible reason for the very late burst in the amplification of raw water samples may be the types of bacteriophages present in the raw water. During periods of rain, the bacteriophages found in raw waters in Fairfax County, Virginia (i.e. water from Occoquan Reservoir, Cherokee Creek, Accotink Creek, etc.) produced medium sized, clear plaques of approximately 2 mm in diameter. After a period of drought, the plaques that appeared on most plates from the raw water samples were very tiny (less than 1 mm in diameter) and very turbid. This indicated that the bacteriophages amplified in dry weather raw water samples were not ΦX174 or T3, but may have been minute plaques of the T even group that exhibited increased burst times when compared to ΦX174 or T3. To eliminate the possibility that the raw water exerted an inhibitory effect on burst time, amplification experiments compared burst times obtained with ΦX174 amplified in TSB and in natural water from Occoquan. Bacteriophage ΦX174 was diluted in TSB and 1.0 ml of this dilution was added to 99 ml of TSB; 1.0 ml was also added to filtered sterilized water from Occoquan. The Occoquan water seeded with ΦX174 was mixed with 3 g TSB and E. coli C to give ~1 x 10^8 cfu/ml. The TSB seeded with ΦX174 also contained ~1 x 10^8 cfu E. coli C/ml. Samples were taken, plated in the usual manner, incubated and the pfu scored. In this experiment, the burst time for ΦX174 in TSB was 30-35 minutes and 35-40 minutes for the seeded Occoquan water. Although the burst time for the ΦX174 in the Occoquan water occurred 5 minutes later than in the TSB, this experiment showed that raw water did not have a significant inhibitory effect on the burst time of a bacteriophage.

The success of the amplification technique is dependent upon the efficiency of the adsorption process during which the bacteriophage adsorbs to and infects a host cell. Percent adsorption was determined for ΦX174 and T3 in the one step growth, single burst and laboratory amplification experiments. The results indicated an inverse relationship between number of bacteriophage present and percent adsorption. The percent adsorption for ΦX174 ranged from 15-66 percent in the one-step and single burst experiments with an increase to 77-89% in the laboratory amplification. Similar results were obtained with T3, for which percent adsorption ranged from 20-92% in the one-step growth and single burst experiments and from 50-92% in the laboratory amplification.

The number of pfu in a raw water sample is unknown and would not usually be determined prior to amplification of a sample, thus percent adsorption could not be determined. The laboratory amplifications in which original pfu present were determined in the ARCAT, demonstrated that percent adsorption at the natural pH of the water samples (pH 6.7 to 7.45) was very high when less than 5 pfu/100 ml were present in the original sample. Based on these finding no alteration of the natural pH of the water samples was deemed necessary.

Optimization of Incubation in Natural Waters--

To determine the optimum incubation time for amplification, a number of experiments were performed on natural water samples from four sources in the Washington, D.C. area. The results of these amplification experiments are presented in Table 27. The water sample from Accotink Creek (9/30) produced no "tiny" pfu, 20 "small" and 6 "medium" pfu when 100 ml were plated at zero time by the regular ARCAT. However, 15 "tiny" pfu/100 ml were present in the amplified sample at 50 minutes. As shown in Table 27, all three morphological types had been amplified by 80 minutes.

TABLE 27. COMPARISON OF NATURAL WATER AMPLIFICATIONS AND ARCAT

Date	Flask Number	Sample Time (minutes)	pfu/100 ml water		
			Tiny	Small	Medium
Accotink Creek Amplification					
9/30	ARCAT*		0	20	6
	1**	50	15	25	5
	2**	70	1205	30	5
	3**	80	1810	210	70
Potomac River Amplification					
10/10	ARCAT*		1	1	0
	1**	50	4040	5	0
	2**	70	16000	95	0
	3**	80	Confluent lysis on the four plates.		
Accotink Creek Amplification					
10/17	ARCAT*		2	6	6
	1**	50	0	40	35
	2**	70	1400	20	50
	3**	80	6	21	11
Accotink Creek Amplification (1:1 Dilution)					
10/17	ARCAT*		0	2	4
	1**	50	10	35	10
	2**	70	1515	0	5
	3**	80	25	75	105
Accotink Creek Amplification (1:1 Dilution)					
10/20	ARCAT*		0	2	0
	1**	50	60	10	5
	2**	70	5050	-	0
	3**	80	2575	-	5
Accotink Creek Amplification (1:3 Dilution)					
10/20	ARCAT*		2	0	0
	1**	50	10	0	0
	2**	70	3660	-	-
	3**	80	14-16,000	-	-

* ARCAT performed on 100 ml water sample.
- Unable to determine due to large number of tiny pfu.
**Calculated value based on assay of 4-5 ml samples by ARCAT.

The second water sample amplified was from the Potomac River (10/10). The regular ARCAT produced 1 "tiny" and 1 "small" pfu in 100 ml of water. The ARCAT count did not correlate well with the amplification count at 50 minutes. These data indicated that the bacteriophage which results in the formation of the "tiny" plaques had already been amplified at 50 minutes. By 70 minutes, it appeared that a second burst of the "tiny" pfu had occurred and a burst of the "small" pfu was indicated.

Raw water from Accotink Creek was used for several amplification experiments. The first experiment (10/17) used undiluted Accotink Creek water. The regular ARCAT produced 2 "tiny", 6 "medium" and 6 "small" pfu/100 ml water sample. Amplification of the "small" and "medium" pfu occurred by 50 minutes. "Tiny" pfu were not observed from flask 1. The flasks used for the 70 and 80 minute samples contained "tiny" pfu as evidenced by the counts of 1400 "tiny" pfu/100 ml and 6 pfu/100 ml from the 70 minute and 80 minute flasks, respectively.

A second Accotink Creek water sample, diluted 1:1 with sterile raw water to reduce the number of pfu/100 ml present in the water, produced 4 "medium" and 2 "small" pfu/100 ml of water in the regular ARCAT. After 50 minutes incubation, amplification occurred as evidenced by detection of 10 "tiny", 35 "small" and 10 "medium" pfu. By 70 minutes 1,515 "tiny" pfu/100 ml were detected and at 80 minutes 75 "small" and 105 "medium" pfu/100 ml were observed.

Accotink Creek water was used for two additional experiments (10/20) in which the water was diluted 1:1 and 3:1 with sterile, raw water. In the first experiment (1:1 dilution), the regular ARCAT produced 2 "small" pfu/100 ml water. By 50 minutes, amplification had begun as demonstrated by the presence of 60 "tiny", 10 "small" and 5 "medium" pfu/100 ml. The ARCAT did not demonstrate the presence of any "tiny" or "medium" pfu but the amplification of the sample demonstrated the presence of these types of pfu in the water sample.

In the second experiment (3:1 dilution with sterile raw water), the regular ARCAT showed the presence of 2 "tiny" pfu/100 ml water sample. The amplification produced only "tiny" pfu, thus, dilution of the water sample appeared to dilute out the "small" and "medium" pfu observed in the Accotink Creek water sample diluted 1:1.

Amplification of well water and water samples from Occoquan reservoir (filtered and raw water) was attempted. The ARCAT on each of these samples produced no plaques for 100 ml plated. Amplification was done with 1×10^8 cfu/ml of E. coli C and samples were plated at intervals up to 80 minutes. Bacteriophage plaques were not detected on any plates.

These amplification experiments demonstrated that when the standard ARCAT procedure (plating of four 5 ml samples) produced no plaques (i.e. less than 5 pfu/100 ml), the amplification procedure could demonstrate the presence of bacteriophages at levels <5 pfu/100 ml. When only 1 or 2 pfu/100 ml of water were present, adsorption took place and amplification occurred by 70-80 minutes.

Amplification experiments performed with multiple 100 ml samples of the same water demonstrated that one morphological type of plaque could be found in one flask but not necessarily in all flasks. This finding indicated that a

particular type of pfu was present in very low numbers in the water sample, so few that in distribution of three 100 ml samples (from 1 liter collected) the few bacteriophages present were not evenly distributed among the flasks. The same conclusion must also be true for pfu determinations in the standard ARCAT when the pfu are present in low numbers and only 20 ml are plated. The amplification technique permits detection of bacteriophage at concentrations below the sensitivity of the standard ARCAT, but the exact number of pfu present in the original water sample must be estimated.

Application of Amplification Techniques to Detection of Very Low Levels of Bacteriophages

Conditions for optimization of adsorption and lytic burst for amplification of bacteriophages present in natural water samples have been determined in these studies. Water samples of 100 ml or 200 ml are suitable for amplification. Nutrient addition is required and may be provided in the form of dry TSB to a final concentration of 3% or as sterile concentrated liquid TSB to the same final concentration. The host E. coli C culture may be a fresh, actively growing culture or frozen E. coli C host thawed at 44.5 $\pm$ 0.2°C for fifteen minutes. A final concentration of ~1 x 10^8 cfu/ml E. coli C host in the amplification flask gives good adsorption, results in an earlier burst time by approximately 10 minutes when compared to that obtained with lower host concentrations and results in amplification of a variety of bacteriophage types. Amplification data for natural water samples indicated that incubation of 50 minutes was not long enough for amplification of the variety of coliphages present on most sample days. Incubation for 80 minutes during amplificaton resulted in confluent lysis or very large numbers of pfu/plate on a number of sample days. In most cases incubation for 70 minutes resulted in optimum amplification of all plaque types present in the water sample.

Natural water samples contain a wide variety of bacteriophages which can produce plaques of different morphologies. Based on data obtained in these studies, two plaque sizes predominated and can be evaluated. The two basic plaque sizes observed were the "tiny" pfu (pinpoint) and the "small" to "medium" pfu. The "tiny" pfu fell into two groups based on burst size and burst time. Group A of the "tiny" pfu produced very large burst sizes with amplification of one or two "tiny" pfu/100 ml resulting in as many as 4,000 pfu/20 ml plated, or 800 pfu/plate (5 ml). Amplification of these "tiny" pfu required approximately 120 minutes of incubation prior to assay. Group B "tiny" pfu required incubation periods of 70-80 minutes for 1 or 2 "tiny" pfu to produce 60 to 235 pfu/plate (5 ml) or 240 to 940 pfu/20 ml plated. Group A "tiny" pfu were observed most frequently during a period of drought and Group B "tiny" pfu were observed in greater numbers when normal rainfall had resumed.

Burst sizes observed for the "small" to "medium" pfu in natural water amplification experiments were considerably smaller than the burst sizes observed for the "tiny" pfu. The presence of 6 "small" to "medium" pfu in 100 ml of the original water sample resulted in an average of 9 pfu/plate (5 ml) or 180 pfu/100 ml of water following amplification. This corresponds to a burst size of thirty which is near the average burst size (24 pfu) determined for the ΦX174 bacteriophage.

The amplification technique can be useful for detection of bacteriophages in natural waters where very low numbers of pfu/100 ml are expected. At levels of $\geq$5 pfu/100 ml, the standard ARCAT is the preferred technique. However, to detect the presence of $\geq$5 bacteriophages per 100 ml, amplification of the natural water sample, performed in parallel with the ARCAT, could provide data on the presence or absence of bacteriophages in the sample. For example, the presence of no "tiny" pfu in the standard ARCAT (20 ml plated) and a count of approximately 300 pfu/plate (5 ml plated) after amplification for 70 or 80 minutes suggests that the number of "tiny" pfu in the original water sample was between 1 and 3. When no "small" or "medium" pfu are detected in the regular ARCAT, amplification can result in an average of 10 pfu/plate or 200 pfu/100 ml, indicating that 3 to 5 pfu were present in the original sample.

The amplification technique can detect very low numbers of bacteriophages in natural waters; however, with the current state-of-the-art, this technique only permits detection and estimation of the number of bacteriophages present in the original sample. For statistical quantitation by this technique, a large number of bacteriophages would have to be correlated according to plaque morphology, burst size and burst time. Amplification should only be used in addition to the standard ARCAT procedure for detection of $\leq$5 pfu/100 ml in a natural water sample.

VERIFICATION STUDIES

Verification of Bacteriophage Recovery

The verification studies with Zeta Plus 60S filters examined the correlation between the recovery of bacteriophages using the ARCAT method and the recovery of bacteriophages using the concentration method. The initial studies on the efficiency of bacteriophage recovery from water samples by the concentration method utilized seeded, sterile raw water samples. In these studies, recovery of 90-100% of total bacteriophages applied to the filter (as determined by ARCAT) was demonstrated in the concentration technique. Recoveries from natural water samples demonstrated a wide range of recovery efficiency. The data comparing the recovery of bacteriophages from natural waters by the concentration technique and the ARCAT method are presented in Table 28.

This project required concentration of very low levels of bacteriophages from water samples. At these low levels, i.e. 1 to 10 pfu/100 ml, small variations in plaque numbers cause great variations in percentages recovered. This variation is illustrated in Table 28 where many of the plaque counts fell in a range of zero to 10 pfu/100 ml as determined in the ARCAT method with corresponding plaque counts from filter concentrated samples showing variation from 12.5% to 240% of the ARCAT plaque count.

Concentration data presented in Table 28 show plaque counts as high as 300% of plaque counts determined by the ARCAT method. This phenomenon has only been noted in natural water samples. During filter concentration of the natural water samples, infected E. coli C cells may be stressed and induced to burst, releasing bacteriophages onto the filter for subsequent elution with the free bacteriophages from the water sample which have also been adsorbed to the filter. Whereas each infected E. coli C cell would be detected as one pfu in the regular ARCAT,

TABLE 28. CORRELATION OF ARCAT AND ZETA PLUS 60S CONCENTRATION METHOD

Date	Sample	ml Filtered	Conc [1]	Ø in 100 ml [2] ARCAT	Ø in Eluate	% Recovered [3]
8/20	Occoquan Raw	750	37.5	3	7	31.1
8/26	Accotink	500	25	6	39	130.0
	Holmes Run	500	25	1	12	240.0
8/27	Accotink	500	25	4	19	95.0
8/28	Potomac Raw	500	25	60	337	112.0
8/29	Accotink	500	25	6	16	53.3
9/2	Cherokee	500	25	8	58	145.0
	Cherokee	100	5	8	15	187.5
9/4	Potomac Raw	500	25	41	143	69.8
	Potomac Raw	100	5	41	22	53.6
	Potomac Settled	1000	50	14	29	20.7
9/8	Cherokee	500	25	3	3	20.0
9/9	Potomac Raw	100	5	27	26	96.3
	Potomac Raw	500	25	27	111	82.2
	Potomac Raw	1000	50	27	162	60.0
	Potomac Settled	100	5	8	1	12.5
	Potomac Settled	500	25	8	15	37.5
	Potomac Settled	1000	50	8	141	176.3
9/15	Potomac Raw	100	5	21	77	366.7
	Potomac Raw	500	25	21	258	245.7
	Potomac Raw	1000	50	21	335	159.5
9/15	Potomac Filtered	500	25	4	22	110.0
9/16	Potomac Raw	100	5	15	10	66.7
	Potomac Raw	100	10	15	15	100.0
	Potomac Settled	100	5	2	1	50.0
	Potomac Settled	100	10	2	1	50.0
9/17	Potomac Raw	100	5	15	21	140.0
	Potomac Raw	100	10	15	10	66.7
	Potomac Settled	100	5	5	5	100.0
	Potomac Settled	100	10	5	8	160.0
9/18	Potomac Raw	100	5	807	620	76.8
	Potomac Raw	100	10	807	391	48.4
	Potomac Settled	100	5	295	295	100.0
	Potomac Settled	100	10	295	159	53.9
9/19	Potomac Raw	100	5	241	239	99.2

(continued)

TABLE 28. (continued)

Date	Sample	ml Filtered	Conc[1]	Ø in 100 ml[2] ARCAT	Ø in Eluate	% Recovered[3]
9/19	Potomac Settled	100	5	72	19	26.4
	Potomac Settled	100	10	72	9	12.5
9/22	Potomac Settled	100	5	3	3	100.0
	Potomac Filtered	100	5	1	2	200.0
9/23	Potomac Raw	200	10	9	40	222.2
	Potomac Settled	200	10	5	6	120.0

1. Concentration factor = total ml filtered divided by the final eluate volume plated (10 ml, 20 ml).
2. Ø - bacteriophages; 100 ml assayed by ARCAT.
3. % recovered is based on Ø applied and Ø recovered.

release of many bacteriophages from each infected cell due to stress caused lysis could result in recoveries such as those found in some natural water samples.

Least squares linear regression analysis was applied to data in Table 28 to determine how well the concentration method of bacteriophage enumeration predicted bacteriophages present in the sample as determined by the ARCAT method. The regression line obtained from the data is shown in Figure 9. The regression was highly significant. The F ratio from the analysis of variance was less than 0.00001 (Table 29). The coefficient of determination was 0.8516 indicating that over 85% of the variation in the bacteriophage determination was related to the concentration of bacteriophages filtered. As illustrated in Table 30, the following equation can be used to predict the number of bacteriophages (Y) applied to the filter based on the number of bacteriophages recovered (X):

$$Y_{pred} = 1.2853X - 3.61$$

The concentration technique can be useful for detection of bacteriophages in natural waters containing very low levels of bacteriophages. As shown in Table 31, filtration of volumes of 100 ml to $\leq$ 500 ml in the concentration technique can detect the one pfu/100 ml that escapes detection in the ARCAT method.

Correlation of Bacteriophages, Fecal Coliforms and Total Coliforms

A variety of natural water sources were sampled and monitored for the presence of bacteriophages and fecal and total coliforms. The bacteriophages were determined by the standard ARCAT method (20 x 5 ml samples plated, no concentration) and the fecal and total coliforms were determined by Standard Methods as described in Section 5. No less than 10% of the total coliforms detected were verified using Lauryl Tryptose Broth and Brilliant Green Broth as described in Section 5.

An areawide drought resulted in substantial reduction of bacteriophages and/or coliforms detected in water samples. Less than one inch of rain on September 18 resulted in a dramatic rise in bacteriophage numbers as well as a rise in total and fecal coliform numbers. However, bacteriophage and total and fecal coliform concentrations had returned to the previous very low levels by the next sampling day.

Correlation of bacteriophages, total and fecal coliforms was not possible for samples that did not show either coliphage or coliforms, therefore, data for analysis were limited to those sample days for which both coliphage and coliform counts were obtained. Data for Potomac raw water (Tables 32 and 33) yielded a correlation coefficient of 0.90 for bacteriophages and total coliforms, a significant correlation indicating 5% chance that no relation exists. Correlation coefficients calculated for Potomac River settled waters (data from Tables 32, 33 and 34) yielded a coefficient of 0.8 for bacteriophages and fecal coliforms with a coefficient of 0.70 for bacteriophages and total coliforms. The bacteriophages and bacteria remaining after filtration yielded a correlation coefficient of 0.75 for bacteriophages and fecal coliforms and a coefficient of 0.614 for bacteriophages and total coliforms.

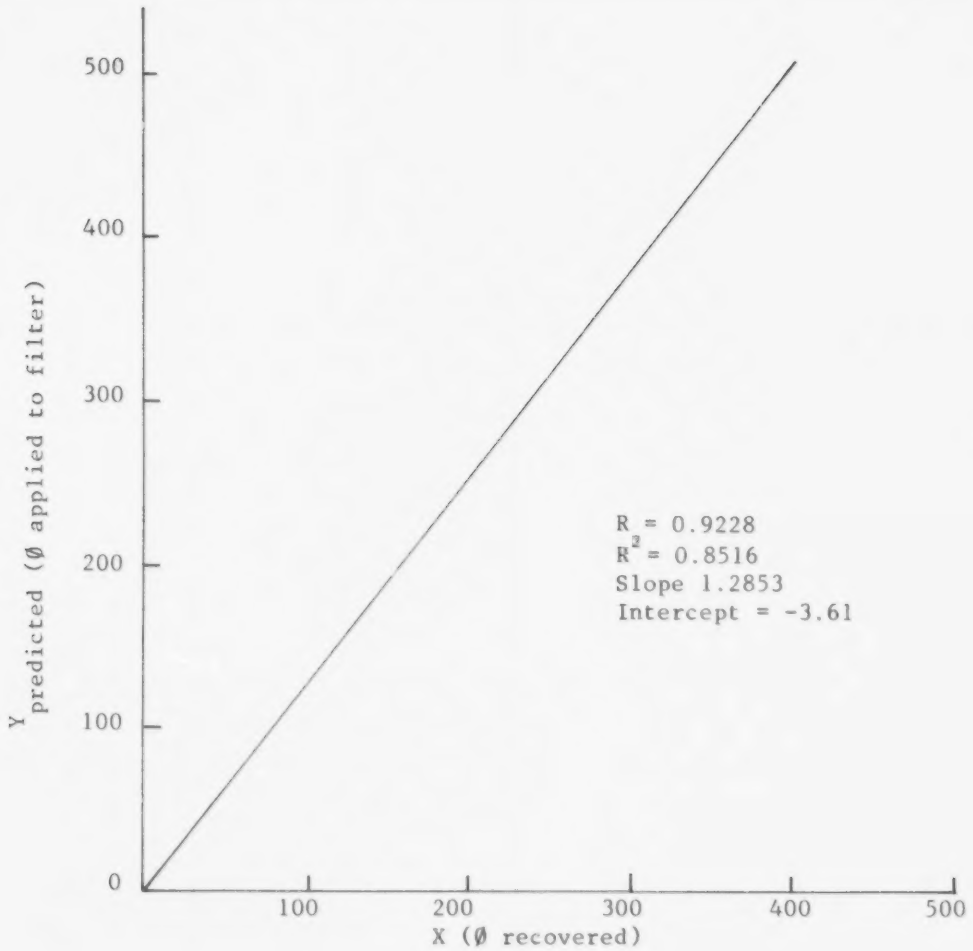


Figure 9. Least Squares Regression Line of Bacteriophages Recovered on Bacteriophages Filtered

TABLE 29. ANALYSIS OF VARIANCE FOR THE REGRESSION OF BACTERIOPHAGE CONCENTRATION ON THE BACTERIOPHAGE CONCENTRATION IN SUSPENSION

Source of Variation	Degrees of Freedom	Sums of Sums	Mean Square	F Ratio
Regression	1	1135673	1135673	200.85
Error	35	197903	5654	
Total	36	1333576		

TABLE 30. PREDICTED COLIPHAGE VERSUS COLIPHAGE APPLIED

X Ø Detected ARCAT Concentration	Y Ø Predicted[1]	Ø Applied[2]
1	-	2, 2
2	-	1
3	0.23	3
5	2.8	5
6	4.1	10
8	6.6	5
10	9.2	15, 15
12	11.75	5
15	15.59	8, 15
16	16.87	30
21	23.3	15
22	24.6	20, 41

Ø - Bacteriophage.

1. The sensitivity of the method is $\geq$ 3 pfu applied to the filter.
2. Calculated value: 100 ml plated in ARCAT x ml filtered/100.

TABLE 31. LOW LEVEL BACTERIOPHAGE RECOVERY BY FILTRATION

Date	Sample	ml Filtered	Concentration	ϕ in 100 ml[1] ARCAT	ϕ in Eluate	TC/100 ml[2]	FC/100 ml[3]
9/2	Accotink	500	25	< 1	17	1.8×10^3	1.5×10^3
	Accotink	100	5	< 1	5	1.8×10^3	1.5×10^3
9/3	Cherokee	100	5	< 1	6	-	-
	Cherokee	500	25	< 1	30	-	-
9/9	Potomac Filtered	500	25	-	2	1	< 1
9/16	Potomac Filtered	500	25	-	1	< 1	< 1
9/17	Potomac Filtered	500	25	1	5	20	6
9/22	Potomac Filtered	100	5	1	2	2.5	1
9/23	Potomac Filtered	200	10	< 1	3	< 1	2

- Not determined

1 ϕ - bacteriophage; 20-5 ml samples assayed by ARCAT

2 TC - total coliforms, not verified

3 FC - fecal coliforms, not verified

EFFECTS OF PHYSICAL TREATMENT ON BACTERIOPHAGES AND BACTERIA

The effects of settling and filtration on bacteriophages are illustrated in Table 32. Bacteriophage numbers are decreased by 60-80% in the settling or flocculation process. Bacteriophages remaining after flocculation were reduced an additional 12-55% by filtration. Overall reduction of bacteriophages present in raw water by physical methods was 93->99.9% on eight of nine sample days.

The effects of physical treatment on total coliforms are shown in Table 33. Samples for one day were drawn simultaneously, thus the raw, settled and filtered samples assayed on a single day did not reflect treatment of a single water sample and can account for a rise in total counts in settled water when low counts are reported for the raw water sample on the same day. The settling process eliminated 63-93% of the total coliforms present, with percentage reduction increasing as total coliforms in raw water samples increased. Total coliforms in raw water samples were reduced 78-99.9% by settling and by filtration.

Physical treatment of raw water resulted in reduction of fecal coliforms as shown in Table 34. As a result of settling, fecal coliforms were generally reduced by 40-85%. Filtration further reduced the fecal coliform count. The cumulative reduction of fecal coliforms by physical processes was 89-99.9%.

The physical processes of settling and filtration are effective means for reducing levels of bacteriophages, and total and fecal coliforms in natural raw waters. Settling resulted in reduction of total coliforms by an average of 79.7%, whereas fecal coliforms were reduced by an average of 71.2% and bacteriophages were reduced by 67.6%. Filtration resulted in additional reduction of all microbial forms. The total reduction achieved by these two physical processes was best for total coliforms with 99% reduction achieved in 3 of 4 samples and fecal coliforms were reduced by $\geq$97% in 5 of 6 samples. Bacteriophages appeared to be removed somewhat less efficiently by these processes with $\geq$92.9% reduction in 8 of 9 samples monitored.

EFFECTS OF DISINFECTANTS ON BACTERIOPHAGES AND BACTERIA

Natural water samples in the Washington, D.C. area during the summer and fall of 1980 generally contained non-detectable or very small numbers of bacteriophages and total and fecal coliforms. In order to determine the effect of disinfectants on bacteriophages and bacteria, a natural water containing large numbers of microorganisms was needed. An enrichment culture of naturally occurring bacteriophages and bacteria from Accotink Creek, Potomac River and Occoquan Reservoir was initiated in the ARC biodisc. TSB (25 ml of 30% TSB) was added to Stage 1 of the Biodisc approximately once a week to provide nutrients for growth and maintenance of the organisms. Samples for treatment were obtained from the outflow of Stage 1 of the Biodisc immediately before treatment was begun. The pH of the Biodisc enrichment culture was consistently between pH 7.0 and pH 7.2. The disinfectants studied were ultraviolet irradiation, chlorine dioxide and ozone.

Ultraviolet Irradiation

The effects of ultraviolet light on bacteria and bacteriophage in the

TABLE 32. EFFECT OF PHYSICAL TREATMENT OF POTOMAC RIVER WATER ON BACTERIOPHAGES (Ø)

Date	A* Ø/100 ml Raw Water	Turbidity†	B* Ø/100 ml Settled Water	Turbidity†	C* Ø/100 ml Filtered Water	Turbidity†	% Decrease A-B/A	% Decrease $\frac{B-C}{A}$	% Decrease A-C/A
9/4	41	25	14	6.1	<1	0.30	65.9	34.0	>99.9
9/10	27	21	8	1.02	0.4	0.19	70.4	28.1	98.5
9/15	21	23	8	3.4	4	0.30	61.9	19.0	81.0
9/16	15	14	2	1.93	0.2	0.12	86.7	12.0	98.7
9/17	15	17	5	2.35	1	0.16	66.7	26.6	93.3
9/18	807	63	295	2.1	19	0.18	63.4	34.3	97.6
9/19	241	27	72	1.57	7	0.22	70.1	27.0	97.1
9/22	14	18	3	1.4	1	0.22	78.6	14.2	92.9
9/23	9	19.5	5	1.4	<1	0.24	44.4	55.5	>99.9

* 20-5 ml samples assayed by ARCAT.

† NTU as measured by the Hach Ratio Turbidimeter, Model 18900, with a range of 0.00-200 NTU.

TABLE 33. EFFECT OF PHYSICAL TREATMENT OF POTOMAC RIVER WATER ON TOTAL COLIFORMS (TC)

Date	A TC/100 ml Raw Water	Turbidity†	B TC/100 ml Settled Water	Turbidity†	C TC/100 ml Filtered Water	Turbidity†	% Decrease A-B/A	% Decrease $\frac{B-C}{A}$	% Decrease A-C/A
9/4	LA	25	10	6.1	1	0.30	-	-	-
9/10	0	21	10	1.02	1	0.19	-	-	-
9/15	0	23	0	3.4	5	0.30	-	-	-
9/16	0	14	20	1.93	0	0.12	-	-	-
9/17	95	18	35	2.35	20	0.16	63.2	15.7	78.9
9/18	4800	63	301	2.1	37	0.18	93.7	5.5	99.2
9/22	625	18	72	1.4	2.5	0.22	88.5	11.1	99.6
9/23	150	19.5	40	1.4	0	0.24	73.3	26.6	>99.9

LA - Laboratory accident.
\- Not able to calculate.

† NTU as measured by the hach Ratio Turbidimeter, Model 18900, with a range of 0.00-200 NTU.

TABLE 34. EFFECT OF PHYSICAL TREATMENT OF POTOMAC RIVER WATER ON FECAL COLIFROMS (FC)

Date	A FC/100 ml Raw Water	Turbidity†	B FC/100 ml Settled Water	Turbidity†	C FC/100 ml Filtered Water	Turbidity†	% Decrease A-B/A	% Decrease $\frac{B-C}{A}$	% Decrease A-C/A
9/4	0	25	13	6.1	0	0.30	-	-	-
9/10	70	21	10	1.02	0	0.19	85.7	14.2	>99.9
9/15	33	23	10	3.4	1	0.30	69.7	27.2	97.0
9/16	108	14	6	1.93	0	0.12	94.4	5.5	>99.9
9/17	55	18	8	2.35	6	0.16	85.4	3.7	89.1
9/18	TNTC*	63	495	2.1	30	0.18	-	-	93.9
9/22	208	18	100	1.4	1	0.22	51.9	47.6	99.5
9/23	100	19.5	60	1.4	2	0.24	40.0	58.0	98.0

* Too numerous to count.

† NTU as measured by the Hach Ratio Turbidimeter, Model 18900, with a range of 0.00-200 NTU.

- Unable to calculate.

enrichment culture were studied. The results are presented in Table 35. Fecal coliforms were inactivated less efficiently than total coliforms and coliphages by exposure to ultraviolet light for 60 seconds. Exposure of samples to ultraviolet light for 10 minutes resulted in >99.8% inactivation of all microbial forms when sample depth was 0.42 cm. Increased depth of sample (0.8 cm) significantly decreased the inactivation of coliphages, total coliforms and fecal coliforms.

Ultraviolet light at wavelengths below 300 nm is absorbed by nucleic acids in bacteria and bacteriophages and can cause mutagenic and lethal changes in the nucleic acids. In general, doses of >7000 uW/sec/cm^2 of UV light (254 nm) are required for inactivation of many bacteria and viruses. The damage to nucleic acids caused by UV light can be repaired by enzymatic and genetic mechanisms, thus allowing microbial cells to escape the effect of the mutations. In addition, UV is absorbed by many substances which then project shadows allowing virions or bacteria to escape inactivation. This repair and shadowing allow significant numbers of bacteria and bacteriophages to survive even after long exposures to UV light (doses of >55 times that normally required for inactivation).

Chlorine Dioxide

The effects of chlorine dioxide on bacteriophages and bacteria in the ARC biodisc enrichment culture were studied. These results are presented in Table 36. At a concentration of 1 mg chlorine dioxide/1, reduction in bacteriophage numbers varied from 60-90 percent. Fecal and total coliforms were reduced by 20 and 30 percent, respectively, and total bacterial counts reduced by 45-50 percent. Chlorine dioxide at 2.5 mg/1 reduced bacteriophage counts by 80-99.99%. Total coliforms were reduced by 75-90%, fecal coliforms by 45->99.99%, and total bacteria by $\geq$95 percent. At 10 mg chlorine dioxide/1, all microbial forms were reduced by $\geq$99 percent.

Chlorine dioxide is being considered as an alternate to chlorine for disinfection of wastewater because it is a more powerful oxidant than chlorine and forms a minimal amount of halogenated compounds. Berg and coworkers (1979) studied chlorine dioxide and chlorine in the disinfection of wastewater and found that chlorine dioxide was a more rapid disinfecting agent and that the use of chlorine dioxide gave a much lower residual concentration. In addition, they concluded that chlorine dioxide was a significantly more effective viricide than chlorine. The brief study of chlorine dioxide disinfection conducted during this program tends to support the findings of Berg et al. (1979).

Ozone

The effects of ozone on bacteriophages and bacteria in the ARC biodisc enrichment culture are summarized in Table 37. Data obtained indicate that bacteriophage counts were not adequately or consistently reduced by treatment at the ozone levels achieved in these studies. With short exposure times, plaque counts increased after ozonation. This rise in plaque numbers may have resulted from ozone induced lysis of infected bacterial cells. Following a ten minute ozone exposure, bacteriophage reductions of 54 to 94% occurred in all tests.

TABLE 35. EFFECT OF ULTRAVIOLET LIGHT ON BACTERIA AND BACTERIOPHAGES

Exposure	Dose μW-sec/cm^2	Ø/100 ml sample	% Reduction
Bacteriophages			
0	0	9.2×10^5	-
10 sec	2,160	6.3×10^5	31.52
60 sec	12,960	3.6×10^4	96.09
10 min	129,600	1.9×10^2	99.98
30 min	388,800	1.5×10^1	99.99
0	0	2.1×10^6	-
60 sec	12,960	3.5×10^4	98.33
10 min	129,600	8.3×10^2	99.96
30 min	388,800	1.5×10^1	99.99
30 min*	388,800	7.5×10^3	99.64
0	0	1.0×10^6	-
60 sec	12,960	2.5×10^3	99.75
10 min	129,600	1.7×10^2	99.98
30 min	388,800	5.0×10^0	99.99
30 min*	388,800	2.3×10^3	99.77
Total Coliforms cfu/100 ml sample			
0	0	5.3×10^7	-
10 sec	2,160	TNTC†	-
60 sec	12,960	8.5×10^6	83.96
10 min	129,600	1.2×10^5	99.77
30 min	388,800	1.3×10^4	99.98
0	0	8.2×10^7	-
60 sec	12,960	6.8×10^5	99.17
10 min	129,600	1.4×10^4	99.98
30 min	388,800	1.6×10^3	99.99
30 min*	388,800	3.1×10^4	99.96
Fecal Coliforms cfu/100 ml sample			
0	0	7.7×10^5	-
10 sec	2,160	1.0×10^6	-
60 sec	12,960	1.8×10^5	76.62
10 min	129,600	3.4×10^3	99.56
30 min	388,800	6.6×10^2	99.91
0	0	1.2×10^5	-
60 sec	12,960	5.0×10^4	58.33
10 min	129,600	5.0×10^2	99.58
30 min	388,800	$<1 \times 10^2$	> 99.92
30 min*	388,800	1.9×10^3	98.42

* Samples were 100 ml (depth of 0.8 cm). All other samples were 50 ml samples (depth of 0.42 cm).

† Too numerous to count.

TABLE 36. CHLORINE DIOXIDE DISINFECTION

Date	Bacteriophages (Ø) T=0 min Ø/ml	Bacteriophages (Ø) T=15 min Ø/ml	Bacteriophages (Ø) % Reduction	Total Coliforms (TC) T=0 min TC/ml	Total Coliforms (TC) T=15 min TC/ml	Total Coliforms (TC) % Reduction	Fecal coliforms (FC) T=0 min FC/ml	Fecal coliforms (FC) T=15 min FC/ml	Fecal coliforms (FC) % Reduction	Total Bacteria (TB) T=0 min TB/ml	Total Bacteria (TB) T=15 min TB/ml	Total Bacteria (TB) % Reduction
1 mg/L Chlorine Dioxide												
10/31	18,800	7,130	62.1							2.4×10^4	1.1×10^4	45.0
11/3	2,600	240	90.77							1.0×10^3	5.0×10^2	50.0
11/4				2,200	1,520	30.9	300	240	20.0			
2.5 mg/L Chlorine Dioxide												
10/30	48,000	0	>99.9									
10/31	18,000	4,064	78.4	17,000	4,000	76.5	3,000	0	>99.9	1.3×10^4	7.7×10^2	94.1
11/3	2,600	90	96.54							2.0×10^4	3.2×10^3	84.0
11/4				2,200	160	92.7	300	160	46.7			
5.0 mg/L Chlorine Dioxide												
10/29	194,800	18	99.99	60,000	0	>99.9						
10/28										4.9×10^5	50/ml	99.99
10/30	48,000	0	>99.9							2.5×10^6	1×10^5	96.00
11/3	2,600	20	99.23				3,000	0	>99.9	1.3×10^4	3.3×10^2	97.5
11/4				2,200	100	95.5	300	60	80.0	1.0×10^3	2.2×10^2	88.0
10 mg/L Chlorine Dioxide												
10/28												
10/29	194,800	0	>99.9	60,000	0	>99.9				2.5×10^6	2.3×10^4	99.1
										4.9×10^5	20	99.99

* No data - problems encountered with the fecal coliform test.

T° - 22-25°C

pH - 7.0-7.2

TABLE 37. EFFECT OF OZONE DISINFECTION

Date	T=0 min. pfu/ml	Exposure T (min)	pfu/ml	O_3 Residual mg/L	% Reduction
			Bacteriophages		
11/10	400	1.0	456	0.00	-
		5.0	184	0.08	54.0
		10.0	24	0.00	94.0
11/11	100	2.5	120	0.07	-
		5.0	140	0.09	-
		10.0	28	0.08	72.0
11/12	300	2.5	172	0.08	42.7
		5.0	162	0.10	46.0
		10.0	128	0.13	57.0
11/13	210	2.5	356	0.12	-
		5.0	268	0.12	-
		10.0	96	0.16	54.3
	T=0 min. cfu/ml		Fecal Coliforms cfu/ml		
11/11	200	2.5	160	0.07	20.0
		5.0	140	0.09	30.0
		10.0	80	0.08	60.0
11/13	260	2.5	24	0.12	90.8
		5.0	20	0.12	92.3
		10.0	16	0.16	93.9
	T=0 min. cfu/ml		Total Coliforms cfu/ml		
11/11	660	2.5	560	0.07	15.0
		5.0	480	0.09	27.0
		10.0	440	0.08	33.0
11/13	3100	2.5	600	0.12	80.6
		5.0	200	0.12	93.5
		10.0	180	0.16	94.2

- Cannot be calculated.
Experiments were performed at temperatures ranging from 22 to 25°C.
Sample pH ranged from pH 7.0 to pH 7.2.

The initial effects of ozone on the total bacteria present indicated a substantial reduction or inhibition of colony forming units amounting to 99.99% after 1 minute of exposure to ozone (Table 38). At subsequent time intervals, the decrease in cfu was less, indicating a recovery in colony forming ability. After ten minutes of exposure to ozone, reduction in total colony forming units was 97%.

The fecal and total coliforms demonstrated a decline in colony forming units with increased exposure to ozone. After ozone treatment for 10 minutes with an ozone residual of 0.08 mg/L, total coliforms were reduced by 60% and fecal coliforms by 33%. With an ozone residual of 0.16 mg/L following a 10 minute exposure to ozone, total coliforms were reduced by 94.2% and fecal coliforms by 93.9%.

Evans (1973) states that the action of ozone as a disinfectant is rapid once the threshold dose has been exceeded, with this dose being a function of the "ozone demand" of the water sample. In a review of ozone as a water and wastewater disinfectant, Venosa (1975) gave data showing that coliform inactivation was proportional to ozone residual with 90.5% inactivation at an O_3 residual of 0.06 mg/L, 94.5% at a residual of 0.08 mg/L and 98% at a residual of 0.16 to 2.0 mg/L. In the ARC studies, a total coliform reduction of 94.2% was achieved with an ozone residual of 0.16 mg/L after 10 minutes exposure to ozone. Carazzone and Vanini (1969) found that 5 minutes of contact at an ozone concentration of 0.5 mg/L was viricidal against coliphage T2. The natural coliphages monitored in the ARC study were decreased by a maximum of 94% by a ten minute exposure to ozone. Hetteche and Ehlback (1955) found that a residual of 0.15 mg ozone/L was sufficient for inactivation of poliomyelitis virus in water. The data indicate the inactivation of different virions by ozone is both concentration and exposure time dependent.

Discussion of Disinfection Results

Ultraviolet irradiation of water samples at very shallow depths (0.42 cm) gave good reduction of total coliforms, fecal coliforms and coliphages following exposure 10 minutes. At a sample depth of 0.8 cm, inactivation of coliforms and coliphages decreased significantly. Particulate matter can result in shading of microorganisms from ultraviolet light and the effects of UV can be modified by enzymatic and genetic repair mechanisms allowing organisms to escape the lethal effects of this treatment. Organisms with comparable amounts of DNA and similar base ratios may have different UV sensitivities because of differences in repair efficiency. Single stranded DNA cannot be repaired by dimer excision or by recombination and is very sensitive to UV whereas double-stranded DNA is more resistant. Because of the microbial repair mechanisms and because UV is absorbed by substances which may cast shadows allowing virions and bacteria to escape the UV light, ultraviolet irradiation does not appear to be well suited to bacterial or viral inactivation.

Chlorine dioxide is a disinfectant with potential as a replacement for chlorine for disinfection of water because it is a more powerful oxidant than chlorine. ARC studies and studies conducted by Berg and coworkers (1979) indicate that chlorine dioxide is more effective as a viricide than chlorine.

TABLE 38. EFFECT OF OZONE ON TOTAL BACTERIA

Date	T=0 min cfu/ml	Exposure T (min)	cfu/ml	O_3 Residual (mg/L)	% Reduction
11/10	1.6×10^5	1.0	1×10^1	0.06	99.99
		5.0	2×10^1	0.08	99.98
		10.0	2.7×10^1	0.00	99.97
11/11	1×10^5	2.5	2.4×10^2	0.07	99.76
		5.0	3×10^2	0.09	99.70
		10.0	2.2×10^3	0.08	97.80
11/12	1×10^5	2.5	2.7×10^2	0.08	99.73
		5.0	1.4×10^3	0.10	98.60
		10.0	1.6×10^3	0.13	98.40
11/13	2.3×10^5	2.5	2.4×10^3	0.12	98.96
		5.0	3×10^3	0.12	98.70
		10.0	2.2×10^3	0.16	99.04

Evans (1973) states that the use of ozone results in disinfection but not necessarily sterilization. Ozone has a half life of only about 25 minutes, thus it lacks a residual effect. The "ozone demand" of the sample determines the threshold dose of ozone required for effective disinfection. Disadvantages of the ozone treatment method include:

- the inability to store ozone because of its short half-life which requires that it be generated locally
- the difficulty in adjusting the ozone application rates to meet the needs of different treatment conditions.

COST ESTIMATES FOR ARCAT AND ZETA PLUS CONCENTRATION METHODS

The concentration of bacteriophages from natural waters with Zeta Plus 60S filters increased the sensitivity of the ARCAT method to the level of 1 pfu/100 ml water sample. The equipment, expendables and personnel required to perform one test are listed in Table 39. Cost comparison of the ARCAT method, the Zeta Plus Concentration method and the Standard Membrane Filter Test are also presented in Table 39. Calculations were made using current prices and the tabular format presented in Pipes, 1978.

TABLE 39. ARCAT AND ZETA PLUS CONCENTRATION TEST COSTS*

Equipment	Cost ($)	Years of Life	Depreciation ($)	% of Use	Chrg to Lab/2400 Tests($)	Category Cost Per Test($)
Freezer	3490.00	20	174.50	20	34.90	
Incubator	486.00	10	48.60	50	24.30	
Water Bath	684.00	10	68.40	100	68.40	
Vortex	102.00	10	10.20	100	10.20	
Balance	97.00	10	9.70	50	4.85	
Water Still	673.00	10	67.30	50	33.65	
Autoclave	1287.00	10	128.70	75	96.53	
Stirring Hot Plate	159.00	10	15.90	100	15.90	
pH Meter	345.00	10	34.50	50	17.25	
Spec 20	743.00	10	74.30	50	37.15	
Test Tubes	113.00/1000	5	22.60	100	22.60	
Test Tubes	104.00/1000	5	20.80	100	20.80	
Pipets 5 ml	137.00/800	5	27.40	100	27.40	
Pipets 25 ml	214.00/400	5	42.80	100	42.80	
	ARCAT Total				$456.73	$ .19
Vacuum Pump	228.00	10	22.80	100	22.80	
Funnels	105.00	2	52.50	100	52.50	
Millipore Filter Apparatus	140.00	10	14.00	100	14.00	
Flask	13.00	2	6.50	100	6.50	
	Zeta Plus Concentration Total				$552.53	$ .23

Expendables	Cost ($)	Charge to Lab/2400 Tests ($) ARCAT	Zeta Plus Conc.	
Petri Dishes	30.00/500	576.00	576.00	
TSB	12.30/454 g	97.53	136.54	
Glycerol	14.00/liter	16.80	16.80	
NA	32.85/454 g			
NaCl	12.67/1000 g	97.68	97.68	
$Sr(NO_3)_2$	21.77/500 g			
NH_4NO_3	7.36/500 g			
Filters	500.00/1000	0.00	1200.00	
	ARCAT Total	$788.02		$ .328
	Zeta Plus Concentration Total		$2027.03	$ .845

*Cost estimates based on 1980 prices and salaries.

(continued)

TABLE 39. (continued)

Personnel			Chrg to Lab/100 Tests($)	Category Cost Per Test($)
ARCAT--				
10 hours	Microbiologist	($10.23/hr)	102.30	
20 hours	Lab Assistant	($6.11/hr)	122.20	
3 hours	Typist	($6.11/hr)	18.33	
		Total	$242.83	$ 2.43
Zeta Plus Concentration--				
10 hours	Microbiologist	($10.23/hr)	102.30	
30 hours	Lab Assistant	($6.11/hr)	183.30	
3 hours	Typist	($6.11/hr)	18.33	
		Total	$303.93	$ 3.04

Total Cost Per Test (1980 dollars)

ARCAT--	
Equipment	0.19
Expendables	0.33
Personnel	2.43
Total	$2.95
Zeta Plus Concentration--	
Equipment	0.23
Expendables	0.85
Personnel	3.04
Total	$4.12

Standard Fecal Coliform Membrane Filter Test Costs (escalated to 1980 dollars) (Pipes, 1978)

Equipment	0.18
Expendables	0.43
Personnel	2.63
Total	$3.24

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